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10      **NOVEL HUMAN GENES RELATING TO RESPIRATORY DISEASES  
AND OBESITY**

**RELATED APPLICATIONS**

15      This application claims the benefit of U.S. Provisional Application Serial Number  
60/146,336 filed July 30, 1999 and U.S. Provisional Application Serial Number  
60/211,749 filed June 14, 2000, the entire teachings of both which are incorporated herein  
by reference.

**FIELD OF THE INVENTION**

20      The present invention relates generally to isolated nucleic acids and the  
classification of the same. The invention more particularly relates to a novel gene and  
novel nucleic acids related to asthma and other respiratory diseases and the classification  
and therapeutic and diagnostic uses of this gene.

25      **BACKGROUND**

Wilkinson *et al.* showed linkage of asthma to markers on human chromosome 12  
(*Genomics*, 53: 251-259 (1998)). In addition, Wilson *et al.* has shown that obesity may  
be linked to asthma ( *Arch. Intern. Med.* 159: 2513-14 (1999)). In particular  
chromosomal region 12q23-qter has been linked to a variety of genetic disorders  
30      including male germ cell tumors, histidinemia, growth retardation with deafness and  
mental retardation, deficiency of Acyl-CoA dehydrogenase, spinal muscular atrophy,

5 Darier disease, cardiomyopathy, Spinocerebellar ataxia-2, brachydactyly,  
Mevalonicaciduria, Hyperimmunoglobulinemia D, Noonan syndrome-1,  
Cardiofaciocutaneous syndrome, spinal muscular atrophy-4, tyrosinemia,  
phenylketonuria, B-cell non-Hodgkin lymphoma, Ulnar-mammary syndrome, Holt-Oram  
syndrome, Scapuloperoneal spinal muscular atrophy, alcohol intolerance, MODY,  
10 Diabetes mellitus, noninsulin-dependent,<sup>2</sup> and diabetes mellitus insulin-dependent (See  
National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/omim/>).  
Although this region appears to contain genes affecting these disorders few genes have  
been discovered. There is a need in the art for identifying specific genes for such  
disorders because they are also associated with obesity and lung disease, particularly  
15 inflammatory lung disease phenotypes such as Chronic Obstructive Lung Disease  
(COPD), Adult Respiratory Distress Syndrome (ARDS), and asthma. Identification and  
characterization of such genetic compositions will make possible the development of  
effective diagnostics and therapeutic means to treat lung related disorders as well as the  
other diseases described herein.

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## SUMMARY OF THE INVENTION

This invention relates to Gene 214 located on chromosome 12q23-qter. Nucleic  
acids comprising all or a part of, or complementary fragments of Gene 214 and cDNA are  
described in various embodiments. Vectors and host cells containing the nucleic acids  
25 herein described are also included in this invention. These nucleic acids can be used in  
therapeutic applications for a multitude of diseases either through the overexpression of a  
recombinant nucleic acid comprising all or a portion of a Gene 214 gene, or by the use of  
these oligonucleotides and genes to modulate the expression of an endogenous gene or  
the activity of an endogenous gene product. Examples of therapeutic approaches include  
30 anti-sense inhibition of gene expression, gene therapy, monoclonal antibodies that

5 specifically bind to the gene products, and the like. In vitro expression of the recombinant gene products can also be obtained.

Diagnostic methods are also described which utilize all or part of the nucleic acids of this invention. Such nucleic acids can be used, for example, as part of diagnostic methods to identify Gene 214 nucleic acids to screen for a predisposition to various genetic diseases. In addition, nucleic acids described herein can be used to identify  
10 chromosomal abnormalities within the chromosomal region 12q23-qter.

Further, this invention identifies various single nucleotide polymorphisms (SNPs) within several of the nucleic acids described herein. Some of these polymorphisms also comprise changes to the polypeptides of the present invention. The SNPs, together with  
15 the wild-type alleles can be used to prepare specific probes for detection of various disease states in an individual. Thus, in one embodiment, this invention provides a method of detecting chromosome abnormalities on chromosome 12q23-qter.

Proteins, polypeptides, and peptides encoded by all or a part of the nucleic acids comprising Gene 214 are included in this invention. Such amino acid sequences are  
20 useful for diagnostic and therapeutic purposes. Further, antibodies can be raised against all or a part of these amino acid sequences for specific diagnostic and therapeutic methods requiring such antibodies. These antibodies can be polyclonal, monoclonal, or antibody fragments.

In a further embodiment, vectors and host cells containing vectors which comprise  
25 all or a portion of the nucleic acid sequences of this invention can be constructed for nucleic acid preparations, including anti-sense, and/or for expression of encoded proteins and polypeptides. Such host cells can be prokaryotic or eukaryotic cells.

Still another embodiment of the invention comprises a method of identifying a protein which is a candidate for being involved in asthma (a "candidate protein").  
30 Candidate proteins are identified by a process comprising (i) identifying a protein in a first individual having the asthma phenotype; (ii) identifying a protein in a second

5 individual not having the asthma phenotype; comparing the protein of the first individual to the protein of the second individual, wherein (a) the protein that is present in the second individual but not the first individual is the candidate protein or (b) the protein that is present in a higher amount in the second individual than in the first individual is the candidate protein or (c) the protein that is present in a lower amount in the second individual than in the first individual is the candidate protein.

10 This invention also includes nonhuman transgenic animals containing one or more of the nucleic acids of this invention for screening and other purposes. Further, knockout nonhuman transgenic animals can be produced wherein one or more endogenous genes or portions of such genes corresponding to the nucleic acids of this invention are replaced by marker genes or are deleted.

#### **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 shows the plot of multipoint LOD score against the map location of the markers along chromosome 12.

20 Figure 2 depicts the STS content of the 12q23-qter BAC RP11-0702C13 containing Gene 214

Figures 3A-3C depict the nucleotide and amino acid sequence of Gene 214a.

Figures 4A-4C depict the nucleotide and amino acid sequence of Gene 214b.

Figures 5A-5C depict the nucleotide and amino acid sequence of Gene 214c.

25 Figures 6A-6D depict the nucleotide and amino acid sequence of Gene 214d.

Figures 7A-7D depict the nucleotide and amino acid sequence of Gene 214e.

Figures 8A-8B show a schematic view of the exons of Gene 214a, 214b, 214c, 214d, and 214e and the corresponding single nucleotide polymorphisms.

Figure 9 shows a Northern Analysis of Gene 214.

30 Figure 10A-10B depicts the nucleic acid sequence of the exons of Gene 214

5 **DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates to Gene 214 nucleic acids comprising genomic DNA within BAC RP11-0702C13, the corresponding cDNA sequences, RNA, fragments of the genomic, cDNA, or RNA nucleic acids comprising 20, 40, 60, 100, 200, 500 or more contiguous nucleotides, and the complements thereof. Closely related variants are also  
10 included as part of this invention, as well as recombinant nucleic acids comprising at least 50, 60, 70, 80, or 90% of the nucleic acids described above which would be identical to a Gene 214 nucleic acids except for one or a few substitutions, deletions, or additions.

Further, the nucleic acids of this invention include the adjacent chromosomal regions of Gene 214 required for accurate expression of the respective gene. In a  
15 preferred embodiment, the present invention is directed to at least 15 contiguous nucleotides of the nucleic acid sequence of any of SEQ ID NO:2 (Figures. 3A-3C), SEQ ID NO:4 (Figures 4A-4C), SEQ ID NO:6 (Figures 5A-5C), SEQ ID NO: 8 (Figures 6A-6D), and SEQ ID NO:10 (Figures 7A-7D). More particularly, embodiments of this invention include the BAC clone containing segments of Gene 214 including RP11-  
20 0702C13. A preferred embodiment is the nucleotide sequence of the BAC clones consisting of SEQ ID NO:1.

This invention further relates to methods using isolated and/or recombinant nucleic acids (DNA or RNA) that are characterized by their ability to hybridize to (a) a nucleic acid encoding a protein or polypeptide, such as a nucleic acid having any of the  
25 sequences of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and SEQ ID NO:10 or (b) a portion of the foregoing (*e.g.*, a portion comprising the minimum nucleotides of the Gene 214 nucleic acid code a functional Gene 214 protein or the minimum number to inhibit an endogenous Gene 214; or by their ability to encode a polypeptide having the amino acid sequence of SEQ ID NO:3, SEQ ID NO:5, SEQ ID  
30 NO:7, SEQ ID NO: 9 and SEQ ID NO: 11 or to encode functional equivalents thereof; *e.g.*, a polypeptide which when incorporated into a cell, has all or part of the activity of a

5 Gene 214 protein, or by both characteristics. A functional equivalent of a Gene 214 protein, therefore, would have a similar amino acid sequence (at least 65% sequence identity) and similar characteristics to, or perform in substantially the same way as Gene 214 protein. A nucleic acid which hybridizes to a nucleic acid encoding a Gene 214 protein or polypeptide, such as SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID  
10 NO: 8, and SEQ ID NO:10 can be double- or single-stranded. Hybridization to DNA such as DNA having the sequence SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and SEQ ID NO:10 includes hybridization to the strand shown or its complementary strand.

In one embodiment, the percent amino acid sequence similarity between a Gene  
15 214 polypeptide such as SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO: 9 and SEQ ID NO: 11, and functional equivalents thereof is at least about 50%. In a preferred embodiment, the percent amino acid sequence similarity between such a Gene 214 polypeptide and its functional equivalents is at least about 65%. More preferably, the percent amino acid sequence similarity between a Gene 214 polypeptide and its  
20 functional equivalents is at least about 75%, and still more preferably, at least about 80%. To determine percent nucleotide or amino acid sequence similarity, sequences can be compared to publicly available sequence databases (National Center for Biotechnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using the blastn2 algorithm (Altsch, *Nucl. Acids Res.*, 25:3389-3402 (1997)). The parameters for a typical search are: E=0.05, v=50, B=50 (where E is the expected probability score cutoff, V is the number of database entries returned in the reporting of the results; and B is the number of sequence alignments returned in the reporting of the results (Altsch *et al*, *J. Mol. Biol.*, 215:403-410 (1990)).

30 Isolated and/or recombinant nucleic acids meeting these criteria comprise nucleic acids having sequences identical to sequences of naturally occurring Gene 214 genes such

5 as Gene 214a, Gene 214b, Gene 214c, Gene 214d, Gene 214e, and portions thereof, or variants of the naturally occurring genes. Such variants include mutants differing by the addition, deletion or substitution of one or more nucleotides, modified nucleic acids in which one or more nucleotides are modified (*e.g.*, DNA or RNA analogs), and mutants comprising one or more modified nucleotides including repeated fragments.

10 Such nucleic acids, including DNA or RNA, can be detected and isolated by hybridization under high stringency conditions or moderate stringency conditions, for example, which are chosen so as to not permit the hybridization of nucleic acids having non-complementary sequences. "Stringency conditions" for hybridizations is a term of art which refers to the conditions of temperature and buffer concentration which permit  
15 hybridization of a particular nucleic acid to another nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first and second may share some degree of complementarity which is less than perfect. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity. "High stringency conditions" and "moderate  
20 stringency conditions" for nucleic acid hybridizations are explained on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., Vol. 1, containing supplements up through Supplement 29, 1995), the teachings of which are hereby incorporated by reference. The exact conditions which determine the stringency of hybridization depend not only on  
25 ionic strength, temperature and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequence, base composition, percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high or moderate stringency conditions can be determined empirically.

30 High stringency hybridization procedures (1) employ low ionic strength and high temperature for washing, such as 0.015 M NaCl/ 0.0015 M sodium citrate, pH 7.0 (0.1x

5   SSC) with 0.1% sodium dodecyl sulfate (SDS) at 50° C; (2) employ during hybridization  
50% (vol/vol) formamide with 5x Denhardt's solution (0.1% weight/volume highly  
purified bovine serum albumin/ 0.1% wt/vol Ficoll/ 0.1% wt/vol polyvinylpyrrolidone),  
50 mM sodium phosphate buffer at pH 6.5 and 5x SSC at 42°C; or (3) employ  
10   hybridization with 50% formamide, 5x SSC, 50 mM sodium phosphate (pH 6.8), 0.1%  
sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml),  
0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2x SSC and 0.1%  
SDS.

By varying hybridization conditions from a level of stringency at which no  
hybridization occurs to a level at which hybridization is first observed, conditions which  
15   will allow a given sequence to hybridize with the most similar sequences in the sample  
can be determined. Preferably the hybridizing sequences will have 60-70% sequence  
identity, more preferably 70-85% sequence identity, and even more preferably 90-100%  
sequence identity.

Exemplary conditions are described in Krause, M.H. and S.A. Aaronson (1991)  
20   *Methods in Enzymology*, 200:546-556. Also, see especially page 2.10.11 in *Current*  
*Protocols in Molecular Biology (supra)*, which describes how to determine washing  
conditions for moderate or low stringency conditions. Washing is the step in which  
conditions are usually set so as to determine a minimum level of complementarity of the  
hybrids. Generally, from the lowest temperature at which only homologous hybridization  
25   occurs, a 1% mismatch between hybridizing nucleic acids results in a 1°C decrease in the  
melting temperature  $T_m$ , for any chosen SSC concentration. Generally, doubling the  
concentration of SSC results in an increase in  $T_m$  of ~17°C. Using these guidelines, the  
washing temperature can be determined empirically for moderate or low stringency,  
depending on the level of mismatch sought.

30   Isolated and/or recombinant nucleic acids that are characterized by their ability to  
hybridize to (a) a nucleic acid encoding a Gene 214 polypeptide, such as the nucleic acids

5 depicted as SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and SEQ ID  
NO:10, b) the complement, (c) or a portion of (a) or (b) (e.g. under high or moderate  
stringency conditions), may further encode a protein or polypeptide having at least one  
function characteristic of a Gene 214 polypeptide, such as protective barrier of the  
respiratory epithelium activity, or binding of antibodies that also bind to non-recombinant  
10 Gene 214 protein or polypeptide. The catalytic or binding function of a protein or  
polypeptide encoded by the hybridizing nucleic acid may be detected by standard  
enzymatic assays for activity or binding (e.g., assays which measure the binding of a  
transit peptide or a precursor, or other components of the translocation machinery).  
Enzymatic assays, complementation tests, or other suitable methods can also be used in  
15 procedures for the identification and/or isolation of nucleic acids which encode a  
polypeptide such as a polypeptide of the amino acid sequences SEQ ID NO:3, SEQ ID  
NO:5, SEQ ID NO:7, SEQ ID NO: 9 and SEQ ID NO: 11, or a functional equivalent of  
these polypeptides. The antigenic properties of proteins or polypeptides encoded by  
hybridizing nucleic acids can be determined by immunological methods employing  
20 antibodies that bind to a Gene 214 polypeptide such as immunoblot, immunoprecipitation  
and radioimmunoassay. PCR methodology, including RAGE (Rapid Amplification of  
Genomic DNA Ends), can also be used to screen for and detect the presence of nucleic  
acids which encode Gene 214-like proteins and polypeptides, and to assist in cloning  
such nucleic acids from genomic DNA. PCR methods for these purposes can be found in  
25 Innis, M.A., *et al.* (1990) *PCR Protocols: A Guide to Methods and Applications*,  
Academic Press, Inc., San Diego, CA., incorporated herein by reference.

It is understood that, as a result of the degeneracy of the genetic code, many  
nucleic acid sequences are possible which encode a Gene 214-like protein or polypeptide.  
Some of these will have little homology to the nucleotide sequences of any known or  
30 naturally-occurring Gene 214-like gene but can be used to produce the proteins and  
polypeptides of this invention by selection of combinations of nucleotide triplets based on

5 codon choices. Such variants, while not hybridizable to a naturally-occurring Gene 214 gene, are contemplated within this invention.

The nucleic acids described herein are used in the methods of the present invention for production of proteins or polypeptides, through incorporation into cells, tissues, or organisms. In one embodiment, DNA containing all or part of the coding  
10 sequence for a Gene 214 polypeptide, or DNA which hybridizes to DNA having the sequence SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and SEQ ID NO:10, is incorporated into a vector for expression of the encoded polypeptide in suitable host cells. The encoded polypeptide consisting of Gene 214, or its functional equivalent is capable of normal activity, such as protecting the respiratory epithelium. The term  
15 "vector" as used herein refers to a nucleic acid molecule capable of replicating another nucleic acid to which it has been linked. A vector, for example, can be a plasmid.

Nucleic acids referred to herein as "isolated" are nucleic acids separated away from the nucleic acids of the genomic DNA or cellular RNA of their source of origin (*e.g.*, as it exists in cells or in a mixture of nucleic acids such as a library), and may have  
20 undergone further processing. "Isolated", as used herein, refers to nucleic or amino acid sequences that are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated. "Isolated" nucleic acids (polynucleotides) include nucleic acids obtained by methods described herein, similar methods or other suitable methods, including essentially pure nucleic acids, nucleic acids  
25 produced by chemical synthesis, by combinations of biological and chemical methods, and recombinant nucleic acids which are isolated. Nucleic acids referred to herein as "recombinant" are nucleic acids which have been produced by recombinant DNA methodology, including those nucleic acids that are generated by procedures which rely upon a method of artificial replication, such as the polymerase chain reaction (PCR)  
30 and/or cloning into a vector using restriction enzymes. "Recombinant" nucleic acids are also those that result from recombination events that occur through the natural

5 mechanisms of cells, but are selected for after the introduction to the cells of nucleic acids designed to allow or make probable a desired recombination event. Portions of the isolated nucleic acids which code for polypeptides having a certain function can be identified and isolated by, for example, the method of Jasin, M., *et al.*, U.S. Patent No. 4,952,501.

10 A further embodiment of the invention is antisense nucleic acids or oligonucleotides which are complementary, in whole or in part, to a target molecule comprising a sense strand, and can hybridize with the target molecule. The target can be DNA, or its RNA counterpart (*i.e.*, wherein T residues of the DNA are U residues in the RNA counterpart). When introduced into a cell, antisense nucleic acids or  
15 oligonucleotides can inhibit the expression of the gene encoded by the sense strand or the mRNA transcribed from the sense strand. Antisense nucleic acids can be produced by standard techniques. See, for example, Shewmaker, *et al.*, U.S. Patent No. 5,107,065.

In a particular embodiment, an antisense nucleic acid or oligonucleotide is wholly or partially complementary to and can hybridize with a target nucleic acid (either DNA or  
20 RNA), wherein the target nucleic acid can hybridize to a nucleic acid having the sequence of the complement of the strand in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and SEQ ID NO:10. For example, an antisense nucleic acid or oligonucleotide can be complementary to a target nucleic acid having the sequence shown as the strand of the open reading frame of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and  
25 SEQ ID NO:10 or nucleic acid encoding a functional equivalent of Gene 214, or to a portion of these nucleic acids sufficient to allow hybridization. A portion, for example a sequence of 16 nucleotides, could be sufficient to inhibit expression of the protein. Or, an antisense nucleic acid or oligonucleotide, complementary to 5' or 3' untranslated regions, or overlapping the translation initiation codon (5' untranslated and translated regions), of  
30 the Gene 214 gene, or a gene encoding a functional equivalent can also be effective. In

5 another embodiment, the antisense nucleic acid is wholly or partially complementary to and can hybridize with a target nucleic acid which encodes a Gene 214 polypeptide.

In addition to the antisense nucleic acids of the invention, oligonucleotides can be constructed which will bind to duplex nucleic acid either in the gene or the DNA:RNA complex of transcription, to form a stable triple helix-containing or triplex nucleic acid to  
10 inhibit transcription and/or expression of a gene encoding Gene 214, or its functional equivalent (Frank-Kamenetskii, M.D. and Mirkin, S.M. (1995) *Ann. Rev. Biochem.* 64:65-95.) Such oligonucleotides of the invention are constructed using the base-pairing rules of triple helix formation and the nucleotide sequence of the gene or mRNA for Gene 214. These oligonucleotides can block Gene 214 -type activity in a number of ways,  
15 including prevention of transcription of the Gene 214 gene or by binding to mRNA as it is transcribed by the gene.

The invention also relates to proteins or polypeptides encoded by the novel nucleic acids described herein. The proteins and polypeptides of this invention can be isolated and/or recombinant. Proteins or polypeptides referred to herein as "isolated" are  
20 proteins or polypeptides purified to a state beyond that in which they exist in cells. In a preferred embodiment, they are at least 10% pure; *i.e.*, most preferably they are substantially purified to 80 or 90% purity. "Isolated" proteins or polypeptides include proteins or polypeptides obtained by methods described *infra*, similar methods or other suitable methods, and include essentially pure proteins or polypeptides, proteins or  
25 polypeptides produced by chemical synthesis or by combinations of biological and chemical methods, and recombinant proteins or polypeptides which are isolated. Proteins or polypeptides referred to herein as "recombinant" are proteins or polypeptides produced by the expression of recombinant nucleic acids.

In a preferred embodiment, the protein or portion thereof has at least one function  
30 characteristic of a Gene 214 protein or polypeptide, for example, protective barrier to the respiratory epithelium activity in the case of Gene 214 analogs, and/or antigenic function

5 (*e.g.*, binding of antibodies that also bind to naturally occurring Gene 214 polypeptide).  
As such, these proteins are referred to as analogs, and include, for example, naturally  
occurring Gene 214 , variants (*e.g.* mutants) of those proteins and/or portions thereof.  
Such variants include mutants differing by the addition, deletion or substitution of one or  
more amino acid residues, or modified polypeptides in which one or more residues are  
10 modified, and mutants comprising one or more modified residues. The variant can have  
“conservative” changes, wherein a substituted amino acid has similar structural or  
chemical properties, *e.g.*, replacement of leucine with isoleucine. More infrequently, a  
variant can have “nonconservative” changes, *e.g.*, replacement of a glycine with a  
tryptophan. Guidance in determining which amino acid residues can be substituted,  
15 inserted, or deleted without abolishing biological or immunological activity can be found  
using computer programs well known in the art, for example, DNASTAR software  
(DNASTAR, Inc., Madison, WI 53715 U.S.A.).

A “portion” as used herein with regard to a protein or polypeptide, refers to  
fragments of that protein or polypeptide. The fragments can range in size from 5 amino  
20 acid residues to all but one residue of the entire protein sequence. Thus, a portion or  
fragment can be at least 5, 5-50, 50-100, 100-200, 200-400, 400-800, or more consecutive  
amino acid residues of a Gene 214 protein or polypeptide, for example, SEQ ID NO:3,  
SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO: 9 and SEQ ID NO: 11, or a variant thereof.

The invention also relates to isolated, synthesized and/or recombinant portions or  
25 fragments of a Gene 214 protein or polypeptide as described above. Polypeptide  
fragments of the enzyme can be made which have full or partial function on their own, or  
which when mixed together (though fully, partially, or nonfunctional alone),  
spontaneously assemble with one or more other polypeptides to reconstitute a functional  
protein having at least one functional characteristic of a Gene 214 protein of this  
30 invention.

5           The invention also concerns the use of the nucleotide sequence of the nucleic acids of this invention to identify DNA probes for Gene 214 genes, PCR primers to amplify Gene 214 genes, nucleotide polymorphisms in Gene 214 genes, and regulatory elements of the Gene 214 genes.

10           Gene 214 was isolated by narrowly defining the region of chromosome 12q23-qter 12q23-qter which was associated with airway hyperresponsiveness and asthma. Gene 214 is also important in other diseases such as obesity and thus, there was a need to identify and isolate the gene.

          To aid in the understanding of the specification and claims, the following definitions are provided.

15           "Disorder region" refers to a portion of the human chromosome 12 bounded by the markers D12S2070 to the 12q telomere. A "disorder-associated" nucleic acid or "disorder-associated" polypeptide sequence refers to a nucleic acid sequence that maps to region 12q23-qter and polypeptides encoded therein. For nucleic acid sequences, this encompasses sequences that are homologous or complementary to the sequence, as well  
20           as "sequence-conservative variants" and "function-conservative variants." For polypeptide sequences, this encompasses "function-conservative variants." Included are naturally-occurring mutations causative of respiratory diseases or obesity, such as but not limited to mutations which cause inappropriate expression (e.g., lack of expression, over-expression, expression in an inappropriate tissue type). "Sequence-conservative" variants  
25           are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. "Function-conservative" variants are those in which a change in one or more nucleotides in a given codon position results in a polypeptide sequence in which a given amino acid residue in a polypeptide has been changed without substantially altering the overall conformation and function of  
30           the native polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic,

5 hydrophobic, and the like). "Function-conservative" variants also include analogs of a given polypeptide and any polypeptides that have the ability to elicit antibodies specific to a designated polypeptide.

"Nucleic acid or "polynucleotide" as used herein refers to purine-and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotide  
10 or mixed polyribo-polydeoxyribo nucleotides. This includes single-and double-stranded molecules, i.e., DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases.

A "coding sequence" or a "protein-coding sequence" is a polynucleotide sequence  
15 capable of being transcribed into mRNA and/or capable of being translated into a polypeptide. The boundaries of the coding sequence are typically determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus.

A "complement" of a nucleic acid sequence as used herein refers to the  
20 "antisense" sequence that participates in Watson-Crick base-pairing with the original sequence.

A "probe" refers to a nucleic acid or oligonucleotide that forms a hybrid structure with a sequence in a target region due to complementarity of at least one sequence in the probe with a sequence in the target region.

Nucleic acids are "hybridizable" to each other when at least one strand of nucleic  
25 acid can anneal to another nucleic acid strand under defined stringency conditions. As is well known in the art, stringency of hybridization is determined, e.g., by (a) the temperature at which hybridization and/or washing is performed, and (b) the ionic strength and polarity (e.g., formamide) of the hybridization and washing solutions, as well as other parameters. Hybridization requires that the two nucleic acids contain  
30 substantially complementary sequences; depending on the stringency of hybridization, however, mismatches may be tolerated. The appropriate stringency for hybridizing

5 nucleic acids depends on the length of the nucleic acids and the degree of complementarily, variables well known in the art.

An "immunogenic component", is a moiety that is capable of eliciting a humoral and/or cellular immune response in a host animal.

10 An "antigenic component" is a moiety that binds to its specific antibody with sufficiently high affinity to form a detectable antigen-antibody complex.

A "sample" as used herein refers to a biological sample, such as, for example, tissue or fluid isolated from an individual (including without limitation plasma, serum, cerebrospinal fluid, lymph, tears, saliva, milk, pus, and tissue exudates and secretions) or from *in vitro* cell culture constituents, as well as samples obtained from e.g., a laboratory  
15 procedure.

"Gene" refers to a DNA sequence that encodes through its template or messenger RNA a sequence of amino acids characteristic of a specific peptide, polypeptide or protein. The term "gene" as used herein with reference to genomic DNA includes intervening, non-coding regions, as well as regulatory regions, and can include 5' and 3'  
20 ends.

"Gene sequence" refers to a DNA molecule, including both a DNA molecule which contains a non-transcribed or non-translated sequence. The term is also intended to include any combination of gene(s), gene fragment(s), non-transcribed sequence(s) or non-translated sequence(s) which are present on the same DNA molecule.

25 A gene sequence is "wild-type" if such sequence is usually found in individuals unaffected by the disease or condition of interest. However, environmental factors and other genes can also play an important role in the ultimate determination of the disease. In the context of complex diseases involving multiple genes ("oligogenic disease"), the "wild type" or normal sequence can also be associated with a measurable risk or  
30 susceptibility, receiving its reference status based on its frequency in the general population.

5           A gene sequence is a "mutant" sequence if it differs from the wild-type sequence. In some cases, the individual carrying such gene has increased susceptibility toward the disease or condition of interest. In other cases, the "mutant" sequence might also refer to a sequence that decreases the susceptibility toward a disease or condition of interest, and thus acting in a protective manner. Also a gene is a "mutant" gene if too much  
10 ("overexpressed") or too little ("underexpressed") of such gene is expressed in the tissues in which such gene is normally expressed, thereby causing the disease or condition of interest.

          A gene sequence is a "variant" sequence if it is substantially similar in structure to either the entire gene or to a fragment of the gene. Both wild-type genes and mutant  
15 genes have variant sequences.

          The sequences of the present invention may be derived from a variety of sources including DNA, cDNA, synthetic DNA, synthetic RNA or combinations thereof. Such sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with  
20 promoter regions or poly (A) sequences. The sequences, genomic DNA or cDNA may be obtained in any of several ways. Genomic DNA can be extracted and purified from suitable cells by means well known in the art. Alternatively, mRNA can be isolated from a cell and used to produce cDNA by reverse transcription or other means.

          "cDNA" refers to complementary or copy DNA produced from an RNA template  
25 by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus, a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector or PCR amplified. This term includes genes from which the intervening sequences have been removed.

          "Recombinant DNA" means a molecule that has been recombined by *in vitro*  
30 splicing/and includes cDNA or a genomic DNA sequence.

5 "Cloning" refers to the use of *in vitro* recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to use methods for generating DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which it can replicate, and for selecting the clone having the target gene  
10 from amongst the recipient host cells.

"cDNA library" refers to a collection of recombinant DNA molecules containing cDNA inserts which together comprise the entire genome of an organism. Such a cDNA library can be prepared by methods known to one skilled in the art and described by, for example, Cowell and Austin, "cDNA Library Protocols," Methods in Molecular Biology  
15 (1997). Generally, RNA is first isolated from the cells of an organism from whose genome it is desired to clone a particular gene.

"Cloning vehicle" refers to a plasmid or phage DNA or other DNA sequence which is able to replicate in a host cell. The cloning vehicle is characterized by one or more endonuclease recognition sites at which such DNA sequences may be cut in a  
20 determinable fashion without loss of an essential biological function of the DNA, which may contain a marker suitable for use in the identification of transformed cells.

"Expression control sequence" refers to a sequence of nucleotides that control or regulate expression of structural genes when operably linked to those genes. These include, for example, the lac systems, the trp system, major operator and promoter  
25 regions of the phage lambda, the control region of fd coat protein and other sequences known to control the expression of genes in prokaryotic or eukaryotic cells. Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host, and may contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements  
30 and/or translational initiation and termination sites.

5 "Expression vehicle" refers to a vehicle or vector similar to a cloning vehicle but which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) an expression control sequence.

10 "Operably linked" means that the promoter controls the initiation of expression of the gene. A promoter is operably linked to a sequence of proximal DNA if upon introduction into a host cell the promoter determines the transcription of the proximal DNA sequence(s) into one or more species of RNA. A promoter is operably linked to a DNA sequence if the promoter is capable of initiating transcription of that DNA sequence.

15 "Host" includes prokaryotes and eukaryotes. The term includes an organism or cell that is the recipient of a replicable expression vehicle.

"Amplification of nucleic acids" refers to methods such as polymerase chain reaction (PCR), ligation amplification (or ligase chain reaction, LCR) and amplification methods based on the use of Q-beta replicase. These methods are well known in the art and described, for example, in U.S. Patent Nos. 4,683,195 and 4,683,202. Reagents and hardware for conducting PCR are commercially available. Primers useful for amplifying sequences from the disorder region are preferably complementary to, and preferably hybridize specifically to, sequences in the 12q23-qter region or in regions that flank a target region therein. Gene 214 generated by amplification may be sequenced directly.

25 Alternatively, the amplified sequence(s) may be cloned prior to sequence analysis.

"Antibodies" refer to polyclonal and/or monoclonal antibodies and fragments thereof, and immunologic binding equivalents thereof, that can bind to asthma proteins and fragments thereof or to nucleic acid sequences from the 12q23-qter region, particularly from the asthma locus or a portion thereof. The term antibody is used both to refer to a homogeneous molecular entity, or a mixture such as a serum product made up of a plurality of different molecular entities. Proteins may be prepared synthetically in a

30

5 protein synthesizer and coupled to a carrier molecule and injected over several months into rabbits. Rabbit sera is tested for immunoreactivity to the protein or fragment. Monoclonal antibodies may be made by injecting mice with the proteins, or fragments thereof. Monoclonal antibodies will be screened by ELISA and tested for specific immunoreactivity with protein or fragments thereof. (Harlow et al, *Antibodies: A*  
10 *Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988.) These antibodies will be useful assays as well as pharmaceuticals.

A nucleic acid or fragment thereof is "substantially homologous" or "substantially similar" to another if, when optimally aligned (with appropriate nucleotide insertions and/or deletions) with the other nucleic acid (or its complementary strand), there is  
15 nucleotide sequence identity in at least about 60% of the nucleotide bases, usually at least about 70%, more usually at least about 80%, preferably at least about 90%, and more preferably at least about 95-98% of the nucleotide bases.

Alternatively, substantial homology or similarity exists when a nucleic acid or fragment thereof will hybridize, under selective hybridization conditions, to another  
20 nucleic acid (or a complementary strand thereof). Selectivity of hybridization exists when hybridization which is substantially more selective than total lack of specificity occurs. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about nine or more nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%.  
25 (See, Kanehisa, [CITE] 1984.) The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will often be over a stretch of at least about 14 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 32 nucleotides, and preferably at least about 36 or more nucleotides.

30 Technical and scientific terms used herein have the meanings commonly understood by one of ordinary skill in the art to which the present invention pertains,

5 unless otherwise defined. Reference is made herein to various methodologies known to those of skill in the art. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full. Standard reference works setting forth the general principles of recombinant DNA technology include Sambrook, J., *et al.*, Molecular  
10 Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, New York (1989); Kaufman, P. B., *et al.*, Eds., Handbook of Molecular and Cellular Methods in Biology and Medicine, CRC Press, Boca Raton (1995); McPherson, M. J., Ed., Directed Mutagenesis: A Practical Approach, IRL Press, Oxford (1991); Jones, J., Amino Acid and Peptide Synthesis, Oxford Science Publications, Oxford (1992); Austen,  
15 B. M. and Westwood, O. M. R., Protein Targeting and Secretion, IRL Press, Oxford (1991); DNA Cloning, Volumes I and II (D.N Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed, 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds. 1984); the series, Methods in Enzymology (Academic Press, Inc.), particularly Vol. 154 and Vol. 155 (Wu and Grossman, eds.); PCR-A Practical Approach (McPherson,  
20 Quirke, and Taylor, eds., 1991); Transcription and Translation, 1984 (Hames and Higgins eds.); Animal Cell Culture, 1986 (R.I. Freshney ed.); Immobilized Cells and Enzymes, 1986 (IRL Press); Perbal, 1984, A Practical Guide to Molecular Cloning; Gene Transfer Vectors for Mammalian Cells, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); Martin J. Bishop, ed., Guide to Human Genome Computing, 2d  
25 Edition, Academic Press, San Diego, CA. (1998); and Leonard F. Peruski, Jr., and Anne Harwood Peruski, The Internet and the New Biology: Tools for Genomic and Molecular Research, American Society for Microbiology, Washington, D.C. (1997). Standard reference works setting forth the general principles of immunology include Sell, S., Immunology, Immunopathology & Immunity, 5th Ed., Appleton & Lange, Publ.,  
30 Stamford, CT (1996); Male, D., *et al.*, Advanced Immunology, 3d Ed., Times Mirror Int'l Publishers Ltd., Publ., London (1996); Stites, D. P., and Terr, A. I., Basic and Clinical

5 Immunology, 7th Ed., Appleton & Lange, Publ., Norwalk, CT (1991); and Abbas, A. K.,  
*et al.*, Cellular and Molecular Immunology, W. B. Saunders Co., Publ., Philadelphia, PA  
(1991). Any suitable materials and/or methods known to those of skill can be utilized in  
carrying out the present invention; however, preferred materials and/or methods are  
described. Materials, reagents and the like to which reference is made in the following  
10 description and examples are obtainable from commercial sources, unless otherwise  
noted.

The nucleic acids of the invention may be isolated directly from cells.  
Alternatively, the polymerase chain reaction (PCR) method can be used to produce the  
nucleic acids of the invention, using either chemically synthesized strands or genomic  
15 material as templates. Primers used for PCR can be synthesized using the sequence  
information provided herein and can further be designed to introduce appropriate new  
restriction sites, if desirable, to facilitate incorporation into a given vector for  
recombinant expression.

The invention also provides vectors comprising the disorder-associated sequences  
20 or derivatives or fragments thereof and host cells for the production of purified proteins.  
A large number of vectors, including plasmid and fungal vectors, have been described for  
replication and/or expression in a variety of eukaryotic and prokaryotic hosts, and may be  
used for gene therapy as well as for simple cloning or protein expression.

Using the information provided in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6,  
25 SEQ ID NO: 8, and SEQ ID NO:10, one skilled in the art will be able to clone and  
sequence all representative nucleic acids of interest, including nucleic acids encoding  
complete protein-coding sequences. It is to be understood that non-protein-coding  
sequences contained within SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:  
8, and SEQ ID NO:10 and the genomic sequence of SEQ ID NO:1 are also within the  
30 scope of the invention. Such sequences include, without limitation, sequences important  
for replication, recombination, transcription and translation. Non-limiting examples

5 include promoters and regulatory binding sites involved in regulation of gene expression, and 5'- and 3'- untranslated sequences (e.g., ribosome-binding sites) that form part of mRNA molecules.

The nucleic acids of the present invention find use as primers and templates for the recombinant production of disorder-associated peptides or polypeptides, for  
10 chromosome and gene mapping, to provide antisense sequences, for tissue distribution studies, to locate and obtain full length genes, to identify and obtain homologous sequences (wild-type and mutants), and in diagnostic applications.

Polypeptides according to the invention are at least five or more residues in length. Preferably, the polypeptides comprise at least about 12, more preferably at least  
15 about 20 and most preferably at least about 30 such residues. Nucleic acids comprising protein-coding sequences can be used to direct the expression of asthma-associated polypeptides in intact cells or in cell-free translation systems. The known genetic code, tailored if desired for more efficient expression in a given host organism, can be used to synthesize oligonucleotides encoding the desired amino acid sequences. The resulting  
20 oligonucleotides can be inserted into an appropriate vector and expressed in a compatible host organism.

The polypeptides of the present invention, including function-conservative variants, may be isolated from wild-type or mutant cells, or from heterologous organisms or cells (e.g., bacteria, fungi, yeast, insect, plant, and mammalian cells) in which a  
25 disorder-associated protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins. The polypeptides can also, advantageously, be made using cell-free protein synthesis systems or by synthetic chemistry. Polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase  
30 synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

5           Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the disorder-associated protein contains an additional sequence tag that facilitates purification. Alternatively, antibodies produced against an disorder-associated protein or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

10           The present invention also encompasses derivatives and homologues of disorder-associated polypeptides. For some purposes, nucleic acid sequences encoding the peptides may be altered by substitutions, additions, or deletions that provide for functionally equivalent molecules, i.e., function-conservative variants.

15           The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

20           Both the naturally occurring and recombinant forms of the polypeptides of the invention can advantageously be used to screen compounds for binding activity. Many methods of screening for binding activity are known by those skilled in the art and may be used to practice the invention. Several methods of automated assays have been developed in recent years so as to permit screening of tens of thousands of compounds in a short period of time. Such high-throughput screening methods are particularly preferred. The use of high-throughput screening assays to test for inhibitors is greatly facilitated by the availability of large amounts of purified polypeptides, as provided by the invention. The polypeptides of the invention also find use as therapeutic agents as well as antigenic components to prepare antibodies.

25           

30

5           The polypeptides of this invention find use as immunogenic components useful as antigens for preparing antibodies by standard methods. It is well known in the art that immunogenic epitopes generally contain at least about five amino acid residues, Ohno et al., 1985, *Proc. Natl. Acad. Sci. USA* 82:2945. Therefore, the immunogenic components of this invention will typically comprise at least five amino acid residues of the sequence of the complete polypeptide chains. Preferably, they will contain at least 7, and most preferably at least about 10 amino acid residues or more to ensure that they will be immunogenic. Whether a given component is immunogenic can readily be determined by routine experimentation. Such immunogenic components can be produced by proteolytic cleavage of larger polypeptides or by chemical synthesis or recombinant technology and are thus not limited by proteolytic cleavage sites. The present invention thus encompasses antibodies that specifically recognize asthma-associated immunogenic components.

          Antibodies according to the present invention include polyclonal and monoclonal antibodies. The antibodies may be elicited in an animal host by immunization with disorder-associated immunogenic components or may be formed by *in vitro* immunization (sensitization) of immune cells. The immunogenic components used to elicit the production of antibodies may be isolated from cells or chemically synthesized. The antibodies may also be produced in recombinant systems programmed with appropriate antibody-encoding DNA. Alternatively, the antibodies may be constructed by biochemical reconstitution of purified heavy and light chains. The antibodies include hybrid antibodies, chimeric antibodies, and univalent antibodies. Also included are Fab fragments, including Fab<sup>1</sup> and Fab(ab)<sup>2</sup> fragments of antibodies.

          These antibodies, whether polyclonal or monoclonal, can be used, e.g., in an immobilized form bound to a solid support by well known methods, to purify the immunogenic components and disorder-associated polypeptides by immunoaffinity chromatography. Antibodies against the immunogenic components can also be used,

5 unlabeled or labeled by standard methods, as the basis for immunoassays, i.e., as diagnostic reagents.

Hybridomas of the invention used to make monoclonal antibodies against the immunogenic components of the invention are produced by well-known techniques. Usually, the process involves the fusion of an immortalizing cell line with a B-  
10 lymphocyte that produces the desired antibody. Alternatively, non- fusion techniques for generating immortal antibody-producing cell lines are possible, and come within the purview of the present invention, e.g., virally-induced transformation, Casali *et al.*, 1986, *Science* 234:476. Immortalizing cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine, and human origin. Most frequently, rat or  
15 mouse myeloma cell lines are employed as a matter of convenience and availability.

Hybridomas are selected by standard procedures, such as HAT (hypoxanthine-aminopterin-thymidine) selection. From among these hybridomas, those secreting the desired antibody are selected by assaying their culture medium by standard immunoassays, such as Western blotting, ELISA (enzyme-linked immunosorbent assay),  
20 Rta (radioimmunoassay), or the like. Antibodies are recovered from the medium using standard protein purification techniques, Tijssen, 1985, *Practice and Theory of Enzyme Immunoassays*, Elsevier, Amsterdam.

#### I. LOCALIZATION OF AN ASTHMA LOCUS ON CHROMOSOME 25 12q23-qter AND THE CHARACTERIZATION OF A CANDIDATE GENE WITHIN THE REGION

To identify genes in the region on 12q23-qter, a set of bacterial artificial chromosome (BAC) clones containing this chromosomal region was identified. The BAC clones served as a template for genomic DNA sequencing and serve as reagents for  
30 identifying coding sequences by direct cDNA selection. Genomic sequencing and direct cDNA selection were used to characterize DNA from 12q23-qter.

5           When a gene has been genetically localized to a specific chromosomal region, the genes in this region can be characterized at the molecular level by a series of steps that include: cloning of the entire region of DNA in a set of overlapping clones (physical mapping), characterization of genes encoded by these clones by a combination of direct cDNA selection, exon trapping and DNA sequencing (gene identification), and  
10       identification of mutations in these genes by comparative DNA sequencing of affected and unaffected members of the kindred and/or in unrelated affected individuals and unrelated unaffected controls (mutation analysis).

          Physical mapping is accomplished by screening libraries of human DNA cloned in vectors that are propagated in a host such as *E. coli*, using hybridization or PCR assays  
15       from unique molecular landmarks in the chromosomal region of interest. To generate a physical map of the disorder region, a library of human DNA cloned in BACs was screened with a set of overgo markers that had been previously mapped to chromosome 12q23-qter by the efforts of the Human Genome Project. Overgos are unique molecular landmarks in the human genome that can be assayed by hybridization. Through the  
20       combined efforts of the Human Genome Project, the location of thousands of overgos on the twenty-two autosomes and two sex chromosomes has been determined. For a positional cloning effort, the physical map is tied to the genetic map because the markers used for genetic mapping can also be used as overgos for physical mapping. By screening a BAC library with a combination of overgos derived from genetic markers,  
25       genes, and random DNA fragments, a physical map comprised of overlapping clones representing all of the DNA in a chromosomal region of interest can be assembled.

          BACs are cloning vectors for large (80 kilobase to 200 kilobase) segments of human or other DNA that are propagated in *E. coli*. To construct a physical map using BACs, a library of BAC clones is screened so that individual clones harboring the DNA  
30       sequence corresponding to a given overgo or set of overgos are identified. Throughout most of the human genome, the overgo markers are spaced approximately 20 to 50

5 kilobases apart, so that an individual BAC clone typically contains at least two overgo markers. In addition, the BAC libraries that were screened contain enough cloned DNA to cover the human genome twelve times over. Therefore, an individual overgo typically identifies more than one BAC clone. By screening a twelve-fold coverage BAC library with a series of overgo markers spaced approximately 50 kilobases apart, a physical map  
10 consisting of a series of overlapping contiguous BAC clones, i.e., BAC "contigs," can be assembled for any region of the human genome. This map is closely tied to the genetic map because many of the overgo markers used to prepare the physical map are also genetic markers.

When constructing a physical map, it often happens that there are gaps in the  
15 overgo map of the genome that result in the inability to identify BAC clones that are overlapping in a given location. Typically, the physical map is first constructed from a set of overgos identified through the publicly available literature and World Wide Web resources. The initial map consists of several separate BAC contigs that are separated by gaps of unknown molecular distance. To identify BAC clones that fill these gaps, it is  
20 necessary to develop new overgo markers from the ends of the clones on either side of the gap. This is done by sequencing the terminal 200 to 300 base pairs of the BACs flanking the gap, and developing a PCR or hybridization based assay. If the terminal sequences are demonstrated to be unique within the human genome, then the new overgo can be used to screen the BAC library to identify additional BACs that contain the DNA from  
25 the gap in the physical map. To assemble a BAC contig that covers a region the size of the disorder region (6,000,000 or more base pairs), it is necessary to develop new overgo markers from the ends of a number of clones.

After building a BAC contig, this set of overlapping clones serves as a template for identifying the genes encoded in the chromosomal region. Gene identification can be  
30 accomplished by many methods. Three methods are commonly used: (1) a set of BACs selected from the BAC contig to represent the entire chromosomal region can be

5 sequenced, and computational methods can be used to identify all of the genes, (2) the BACs from the BAC contig can be used as a reagent to clone cDNAs corresponding to the genes encoded in the region by a method termed direct cDNA selection, or (3) the BACs from the BAC contig can be used to identify coding sequences by selecting for specific DNA sequence motifs in a procedure called exon trapping. The present

10 invention includes Gene 214 identified by the first two methods.

To sequence the entire BAC contig representing the disorder region, a set of BACs can be chosen for subcloning into plasmid vectors and subsequent DNA sequencing of these subclones. Since the DNA cloned in the BACs represents genomic DNA, this sequencing is referred to as genomic sequencing to distinguish it from cDNA  
15 sequencing. To initiate the genomic sequencing for a chromosomal region of interest, several non-overlapping BAC clones are chosen. DNA for each BAC clone is prepared, and the clones are sheared into random small fragments which are subsequently cloned into standard plasmid vectors such as pUC18. The plasmid clones are then grown to propagate the smaller fragments, and these are the templates for sequencing. To ensure  
20 adequate coverage and sequence quality for the BAC DNA sequence, sufficient plasmid clones are sequenced to yield three-fold coverage of the BAC clone. For example, if the BAC is 100 kilobases long, then phagemids are sequenced to yield 300 kilobases of sequence. Since the BAC DNA was randomly sheared prior to cloning in the phagemid vector, the 300 kilobases of raw DNA sequence can be assembled by computational  
25 methods into overlapping DNA sequences termed sequence contigs. For the purposes of initial gene identification by computational methods, three-fold coverage of each BAC is sufficient to yield twenty to forty sequence contigs of 1000 base pairs to 20,000 base pairs.

The sequencing strategy employed in this invention was to initially sequence  
30 "seed" BACs from the BAC contig in the disorder region. The sequence of the "seed" BACs was then used to identify minimally overlapping BACs from the contig, and these

5 were subsequently sequenced. In this manner, the entire candidate region can be sequenced, with several small sequence gaps left in each BAC. This sequence serves as the template for computational gene identification. One method for computational gene identification is to compare the sequence of BAC contig to publicly available databases of cDNA and genomic sequences, e.g. unigene, dbEST, genbank. These comparisons are  
10 typically done using the BLAST family of computer algorithms and programs (Altschul et al, *J. Mol. Biol.*, 215:403- 410 (1990)). The BAC sequence can also be translated into protein sequence, and the protein sequence can be used to search publicly available protein databases, using a version of BLAST designed to analyze protein sequences (Altschul et al, *Nucl. Acids Res.*, 25:3389-3402 (1997)). Another method is to use  
15 computer algorithms such as MZEF (Zhang, *Proc. Natl. Acad. Sci.*, 94:565-568 (1997)), GRAIL (Uberbacher et al, *Methods Enzymol.*, 266:259- 281 (1996)), and Genscan (Burge and Karlin, *J. Mol. Biol.*, 268:78-94) which predicts the location of exons in the sequence based on the presence of specific DNA sequence motifs that are common to all exons, as well as the presence of codon usage typical of human protein encoding sequences.

20 In addition to identifying genes by computational methods, genes were also identified by direct cDNA selection (Del Mastro and Lovett, *Methods in Molecular Biology*, Humana Press Inc., NJ (1996)). In direct cDNA selection, cDNA pools from tissues of interest are prepared, and BACs from the candidate region are used in a liquid hybridization assay to capture the cDNAs which base pair to coding regions in the BAC.  
25 In the methods described herein, the cDNA pools were created from several different tissues by random priming and oligo dT priming the first strand cDNA from polyA RNA, synthesizing the second strand cDNA by standard methods, and adding linkers to the ends of the cDNA fragments. The linkers are used to amplify the cDNA pools. BAC clones from the disorder region identified by screening the RPCI-11 BAC library (P.  
30 deJong, Russell Park Cancer Institute) were used as a template for initiating DNA synthesis to create a biotin labeled copy of BAC DNA. The biotin labelled copy of the

5 BAC DNA is then denatured and incubated with an excess of the PCR amplified, linked  
cDNA pools which have also been denatured. The BAC DNA and cDNA are allowed to  
anneal in solution, and heteroduplexes between the BAC and the cDNA are isolated using  
streptavidin coated magnetic beads. The cDNAs that are captured by the BAC are then  
amplified using primers complimentary to the linker sequences, and the  
10 hybridization/selection process is repeated for a second round. After two rounds of direct  
cDNA selection, the cDNA fragments are cloned, and a library of these direct selected  
fragments is created.

The cDNA clones isolated by direct selection are analyzed by two methods. Since  
a pool of BACs from the disorder region is used to provide the genomic target DNA  
15 sequence, the cDNAs must be mapped to BAC genomic clones to verify their  
chromosomal location. This is accomplished by arraying the cDNAs in microtiter dishes,  
and replicating their DNA in high density grids. Individual genomic clones known to  
map to the region are then hybridized to the grid to identify direct selected cDNAs  
mapping to that region. cDNA clones that are confirmed to correspond to individual  
20 BACs are sequenced. To determine whether the cDNA clones isolated by direct selection  
share sequence identity or similarity to previously identified genes, the DNA and protein  
coding sequences are compared to publicly available databases using the BLAST family  
of programs.

The combination of genomic DNA sequence and cDNA sequence provided by  
25 BAC sequencing and by direct cDNA selection yields an initial list of putative genes in  
the region. The genes in the region were all candidates for the asthma locus. To further  
characterize each gene, Northern blots were performed to determine the size of the  
transcript corresponding to each gene, and to determine which putative exons were  
transcribed together to make an individual gene. For Northern blot analysis of each gene,  
30 probes were prepared from direct selected cDNA clones or by PCR amplifying specific  
fragments from genomic DNA, cDNA or from the BAC encoding the putative gene of

5 interest. The Northern blots gave information on the size of the transcript and the tissues in which it was expressed. For transcripts which were not highly expressed, it was sometimes necessary to perform a reverse transcription PCR assay using RNA from the tissues of interest as a template for the reaction.

Gene identification by computational methods and by direct cDNA selection  
10 provides unique information about the genes in a region of a chromosome. When genes are identified, then it is possible to examine different individuals for mutations in each gene. Variants in gene sequences between individuals can be inherited allelic differences or can arise from mutations in the individuals. Gene sequence variants are clinically important in that they can affect drug action on such gene. Most drugs elicit a safe  
15 response in only a fraction of individuals, and drugs are commonly administered to patients with no certainty that they will be safe and effective. Many important drugs are effective in only 30-40% of patients for whom the drug is prescribed, and virtually all drugs cause adverse events in some individuals. Identification of mutations in disorder genes in different individuals will enable a correlation between the safety and efficacy of  
20 drug therapies used to treat lung diseases and the genotypes of the treated individuals. This correlation enables health care providers to prescribe a drug regimen which is most appropriate for the individual patient rather than trying different drug regimens in turn until a successful drug is identified. Identification of variants in disorder genes will also have a benefit during the development of new drugs for the treatment of lung diseases, as  
25 the ability to correlate genetic variation with the efficacy of new candidate drugs will enhance lead optimization and increase the efficiency and success rate of new drug approvals.

#### A. FAMILY COLLECTION

30 A critical component of any disease gene search is the careful selection and phenotyping of family resources. The family collection utilized in this study consists of

5 421 Caucasian affected sibling ("sib") pairs families collected in the United States and the United Kingdom, as well as an additional 63 Caucasian families from the United Kingdom collected under different ascertainment criteria.

The affected sibling (or "sib") pair families in the United States collection were Caucasian families with two affected siblings that were identified through both private  
10 practice and community physicians. Advertising was also used to identify candidates. A total of 98 families were collected in Kansas, Nebraska, and Southern California. In the United Kingdom collection, 323 families were identified through physicians' registers in a region surrounding Southampton and including the Isle of Wight.

Families were included in the study if they met all of the following criteria: (1) the  
15 biological mother and biological father were Caucasian and agreed to participate in the study, (2) at least two biological siblings were alive, each with a current physician diagnosis of asthma, and 5 to 21 years of age, and (3) the two siblings were currently taking asthma medications on a regular basis. This included regular, intermittent use of inhaled or oral bronchodilators and regular use of cromolyn, theophylline, or steroids.

20 Families were excluded from the study if they met any one of the following criteria: (1) both parents were affected (i.e., with a current diagnosis of asthma, having asthma symptoms, or on asthma medications at the time of the study), or (2) any of the siblings to be included in the study was less than 5 years of age, or (3) any asthmatic family member to be included in the study was taking beta-blockers at the time of the  
25 study or (4) any family member had congenital or acquired pulmonary disease at birth (e.g. cystic fibrosis) history of serious cardiac disease (myocardial infarction) or any history of serious pulmonary disease (e.g. emphysema) or (5) pregnant.

An additional 63 families from the United Kingdom were utilized from an earlier collection effort with different ascertainment criteria. These families were recruited  
30 either: 1) without reference to asthma and atopy or 2) by having at least one family member or at least two family members affected with asthma. The randomly ascertained

5 samples were identified from general practitioner registers in the Southampton area. For the families with affected members, the probands were recruited from hospital based clinics in Southampton. The phenotypic and genotypic data information for 17 markers for 21 of these 63 families was obtained from the website: <http://cedar.genetics.soton.ac.uk/pub/PROGRAMS/BETA/data/bet12.ped>.

## 10 B. GENOME SCAN

In order to identify chromosomal regions linked to asthma, the inheritance pattern of alleles from genetic markers spanning the genome was assessed on the collected family resources. As described above, combining these results with the segregation of the asthma phenotype in these families allows the identification of genetic markers that are tightly linked to asthma, thus providing an indication of the location of genes predisposing affected individuals to asthma. The following discussion describes the protocol used to assess the genotypes of the collected population using genetic markers spanning the entire genome.

20 Genotypes of PCR amplified simple sequence microsatellite genetic linkage markers were determined using ABI model 377 Automated Sequencers. Microsatellite markers comprising a variation of a human linkage mapping panel as released from the Cooperative Human Linkage Center (CHLC), also known as the Weber lab screening set version 8, were obtained from Research Genetics Inc. (Huntsville, Al) in the fluorescent dye-conjugated form (Dubovsky et al., *Hum. Mol. Genet.* Mar; 4(3):449-452 (1995)).

25 Our variation of the Weber 8 screening set consists of 529 markers with an average spacing of 6.87 cM (autosomes only) and 6.98 cM (all chromosomes). Eighty-nine percent of the markers consist of either tri- or tetra-nucleotide microsatellites. In addition, there exist no gaps in chromosomal coverage greater than 17.5 cM.

30 Study subject genomic DNA (5µl; 4.5ng/µl) was amplified in a 10 µl PCR reaction using AmpliTaq Gold DNA polymerase (0.225 U) and containing the final

5 reaction components: 1X PCR buffer (80 mM  $(\text{NH}_4)_2\text{SO}_4$ , 30 mM Tris-HCl (pH 8.8),  
0.5% Tween-20), 200 $\mu\text{M}$  each dATP, dCTP, dGTP and dTTP, 1.5-3.5  $\mu\text{M}$   $\text{MgCl}_2$  and  
250  $\mu\text{M}$  forward and reverse PCR primers. PCR reactions were set up in 192 well plates  
(Costar) using a Tecan Genesis 150 robotic workstation equipped with a refrigerated  
deck. PCR reactions were overlaid with 20  $\mu\text{l}$  mineral oil, and thermocycled on an MJ  
10 Research Tetrad DNA Engine equipped with four 192 well heads under the following  
conditions: 92°C for 3 min, 6 cycles of 92°C 30 sec, 56°C 1 min, 72°C 45 sec, followed  
by 20 cycles of 92°C 30 sec, 55°C 1 min, 72°C 45 sec and a 6 min incubation at 72°C.  
PCR products of 8-12 microsatellite markers were subsequently pooled using a Tecan  
Genesis 200 robotic workstation into two 96 well microtitre plates (2.0  $\mu\text{l}$  PCR product  
15 from TET and FAM labeled markers, 3.0 l HEX labeled markers) and brought to a final  
volume of 25 $\mu\text{l}$  with  $\text{H}_2\text{O}$ . 1.9  $\mu\text{l}$  of pooled PCR product was transferred to a loading  
plate and combined with 3.0  $\mu\text{l}$  loading buffer (loading buffer is 2.5 l formamide/blue  
dextran (9.0 mg/ml), 0.5  $\mu\text{l}$  GS-500 TAMRA labeled size standard, Perkin- Elmer/ABI  
division). Samples were denatured in the loading plate for 4 min at 95°C, placed on ice  
20 for 2 min, and electrophoresed in a 5% denaturing polyacrylamide gel (FMC on the ABI  
377XL). Samples (0.8  $\mu\text{l}$ ) were loaded using an 8 channel Hamilton Syringe pipettor.

Each gel consisted of 62 study subjects and 2 control subjects (CEPH parents ID  
#1331-01 and 1331-02, Coriell Cell Repository, Camden, NJ). Genotyping gels were  
scored in duplicate by investigators blind to patient identity and affection status using  
25 GENOTYPER analysis software V 1.1.12 (ABI Division, Perkin Elmer Corporation).  
Nuclear families were loaded onto the gel with the parents flanking the siblings to  
facilitate error detection. Data with allele peak amplitude less than 100, as detected by  
GENESCAN analysis software V 2.0.2 (ABI Division, Perkin Elmer Corporation), were  
either left unscored or rerun.

30 The final tables obtained from the Genotyper output for each gel analysed were  
imported into a Sybase Database. Allele calling (binning) was performed using the

5 SYBASE version of the ABAS software (Ghosh et al, *Genome Research* 7:165-178  
(1997)). Offsize bins were checked manually and incorrect calls were corrected or  
blanked. The binned alleles were then imported into the program MENDEL (Lange et  
al., *Genetic Epidemiology*, 5, 471(1988)) for inheritance checking using the USERM13  
subroutine (Boehnke et al, *AM. J. Hum. Genet.* 48:22-25 (1991)). Non-inheritance was  
10 investigated by examining the genotyping traces and once all discrepancies were  
resolved, the subroutine USERM13 was used to estimate allele frequencies.

### C. LINKAGE ANALYSIS

Linkage analysis is possible because of the nature of inheritance of chromosomes  
15 from parents to offspring. During meiosis, the two parental homologues pair to guide  
their proper separation to daughter cells. While they are lined up and paired, the two  
homologues exchange pieces of the chromosomes, in an event called "crossing over" or  
"recombination." The resulting chromosomes contain parts that originate from both  
parental homologues. The closer together two sequences are on the chromosome, the less  
20 likely that a recombination event will occur between them, and the more closely linked  
they are. Data obtained from the different families are combined and analyzed together by  
a computer using statistical methods. The result is information indicating the evidence for  
linkage between the genetic markers used and a disease susceptibility locus. A  
recombination frequency of 1% is equivalent to approximately 1 map unit, a relationship  
25 that holds up to frequencies of about 20% or 20 cM. Furthermore, 1 centiMorgan (cM) is  
roughly equivalent to 1,000 kb of DNA.

The entire human genome is 3,300 cM long. In order to find an unknown disease  
gene within 5-10 cM of a marker locus, the whole human genome can be searched with  
roughly 330 informative marker loci spaced at approximately 10 cM intervals (Botstein et  
30 al, *Am. J. Hum. Genet.*, 32:314-331 (1980)). The reliability of linkage results is  
established by using a number of statistical methods. The methods most commonly used

5 for the detection by linkage analysis of oligogenes involved in the etiology of a complex trait are non-parametric or model-free methods which have been implemented into the computer programs MAPMAKER/SIBS (Kruglyak L & Lander ES, Am J Hum Genet 57:439-454, 1995) and GENEHUNTER (Kruglyak L et al., Am J Hum Genet 58:1347-1363,1996). Linkage analysis is performed by typing members of families with multiple  
10 affected individuals at a given marker locus and evaluating if the affected members (excluding parent-offspring pairs) share alleles at the marker locus that are identical by descent (IBD) more often than expected by chance alone. As a result of the rapid advances in mapping the human genome over the last few years, and concomitant improvements in computer methodology, it has become feasible to carry out linkage  
15 analyses using multi-point data. Multi-point analysis provides a simultaneous analysis of linkage between the trait and several linked genetic markers, when the recombination distance among the markers is known. A LOD score statistic is computed at multiple locations along a chromosome to measure the evidence that a susceptibility locus is located nearby. A LOD score is the logarithm base 10 of the ratio of the likelihood that a  
20 susceptibility locus exists at a given location to the likelihood that no susceptibility locus is located there. By convention, when testing a single marker, a total LOD score greater than +3.0 (that is, odds of linkage being 1,000 times greater than odds of no linkage) is considered to be significant evidence for linkage.

Multi-point analysis is advantageous for two reasons. First, the informativeness  
25 of the pedigrees is usually increased. Each pedigree has a certain amount of potential information, dependent on the number of parents heterozygous for the marker loci and the number of affected individuals in the family. However, few markers are sufficiently polymorphic as to be informative in all those individuals. If multiple markers are considered simultaneously, then the probability of an individual being heterozygous for at  
30 least one of the markers is greatly increased. Second, an indication of the position of the disease gene among the markers may be determined. This allows identification of

5 flanking markers, and thus eventually allows identification of a small region in which the disease gene resides.

For the initial linkage analysis, the phenotype and asthma affection status were defined by a patient described above who answered the following questions in the affirmative: (i) have you ever had asthma, (ii) do you have a current physician's  
10 diagnosis of asthma, and (iii) are you currently taking asthma medications? Medications include inhaled or oral bronchodilators, cromolyn, theophylline or steroids.

The distribution of the number of genotyped affected siblings was as follows: 88.7% of the families had 2 siblings, 10.9% had 3 siblings and 0.5% had 4 siblings. Ninety eight families were ascertained in the US and 386 in the UK.

15 Allele sharing methods, implemented in the MAPMAKER/SIBS(Kruglyak L & Lander ES, Am J Hum Genet 57:439-454, 1995), were used on our sample of affected sibling pairs. Multipoint linkage analyses were performed using 54 polymorphic markers spanning a 162 cM region on both arms of chromosome 12. The map location and distances between markers were obtained from the genetic maps published by the  
20 Marshfield medical research foundation (<http://www.marshmed.org/genetics/>). Ambiguous order in the Marshfield map was resolved using the program MULTIMAP (Matisse TC et al., Nature Genet 6:384-390, 1994) on the 46.

Figure 1 displays the multipoint LOD score against the map location of markers along chromosome 12. A Maximum LOD Score (MLS) of 2.9 was obtained at location  
25 161.7 cM, 1.0 cM distal to markers D12S97 and D12S1045. An excess sharing by descent (Identity By Descent, IBD=2) of 0.31 was observed at the maximum LOD score. Table 1 lists the single and multipoint LOD scores at each marker.

These data suggest that chromosome 12 is a location that may contain a gene or genes involved in asthma and diseases thereof.

30 **Table 1: Chromosome 12 Linkage Analysis**

5

Marker	Distance	Two-point	Multipoint
D12S372	6.4	0.0	0.0
GATA49D12	17.7	0.0	0.0
D12S77	20.3	0.0	0.0
D12S391	26.2	0.0	0.0
D12S358	26.2	0.0	0.0
D12S364	30.6	0.2	0.0
D12S373	36.1	0.0	0.0
D12S1042	48.7	0.0	0.0
GATA91H06	56.3	0.0	0.0
D12S368	66.0	0.2	0.3
D12S398	68.2	0.2	0.4
D12S83	75.2	1.1	0.0
D12S1294	78.1	0.0	0.0
IFNgama	80.4	0.0	0.0
D12S375	80.5	0.3	0.0
D12S43	80.5	0.3	0.0
D12S1052	83.2	0.0	0.0
D12S92	83.2	1.0	0.0
D12S326	86.4	0.1	0.1
D12S64	89.4	0.0	0.2
D12S379	93.7	0.0	0.1
D12S311	94.5	0.1	0.0
D12S82	95.0	0.1	0.1
D12S819	95.0	0.0	0.1
D12S1064	95.0	0.0	0.0
D12S95	96.1	0.2	0.2
D12S829	97.2	0.1	0.6
D12S1706	104.1	0.6	0.4
D12S1300	104.1	0.2	0.3
D12S1727	107.2	0.0	0.1
D12S1607	107.9	0.0	0.1

IGF1	109.5	0.0	0.0
PAH	109.5	0.0	0.0
D12S360	111.3	0.0	0.0
D12S338	111.9	0.0	0.0
D12S78	111.9	0.0	0.0
D12S811	120.7	0.1	0.3
D12S1341	123.0	0.0	0.5
NOS1	123.1	0.1	0.4
D12S2070	125.3	0.2	0.7
D12S366	133.3	1.2	1.7
D12S1619	134.5	0.8	1.8
D12S385	135.1	2.0	1.6
PLA2G1B	136.8	0.9	1.4
D12S395	136.8	2.1	1.5
D12S300	140.2	0.9	1.7
D12S342	144.8	1.6	2.2
D12S324	147.2	1.3	1.4
D12S2078	149.6	0.9	1.9
D12S1659	155.9	0.3	1.6
D12S97	160.7	0.9	2.7
D12S1045	160.7	3.0	2.8
D12S392	165.7	1.1	2.3
D12S357	168.8	0.8	1.1

5

#### D. LINKAGE RESULTS

The linkage results for chromosome 12 described above were used to delineate a candidate region for disorder-associated gene(s) located on chromosome 12. Gene discovery efforts were initiated in a ~43 cM interval from marker D12S2070 to the 12q telomere, representing a 99% confidence interval. All genes known to map to this interval were considered as candidates. The discovery of novel genes using direct cDNA

10

5 selection focused on a ~15 cM region approximately between markers D12S1609 and D12S357.

The following section describes details of the efforts to generate cloned coverage of the disorder gene region on chromosome 12, i.e., construction of a BAC contig spanning the region. There are two primary reasons for this: 1) to provide genomic clones for DNA sequencing; analysis of this sequence provides information about the gene content of the region, and 2) to provide reagents for direct cDNA selection; this provides additional information about novel genes mapping to the interval. The physical map consists of an ordered set of molecular landmarks, and a set of bacterial artificial chromosome (BAC, Kim, U.-J., et al., (1996), *Genomics* 34, 213-218 and Shizuya, H., et al., (1992). *Proc. Natl. Acad. Sci. USA* 89, 8794-8797) clones that contain the disorder gene region from chromosome 12q23-qter.

Figure 2 depicts the STS content of BAC RP11-0702113 in 12q23-qter. Gene 214 is located within this BAC as indicated at the top of the figure. Markers used to screen the RPCI-11 BAC library (P. deJong - Roswell Park Cancer Institute) are shown vertically above the solid black horizontal line. The following steps were performed:

1. Map Integration. Various publicly available mapping resources were utilized to identify existing STS markers (Olson et al, (1989), *Science*, 245:1434-1435) in the 12q23-qter region. Resources included the Genome Database (GDB, <http://gdbwww.gdb.org/>), Genethon ([http://www.genethon.fr/genethon\\_en.html](http://www.genethon.fr/genethon_en.html)), Marshfield Center for Medical Genetics (<http://www.marshmed.org/genetics/>), the Whitehead Institute Genome Center (<http://www-genome.wi.mit.edu/>), GeneMap98, dbSTS and dbEST (NCBI, <http://www.ncbi.nlm.nih.gov/>), the Sanger Centre (<http://www.sanger.ac.uk/>), and the Stanford Human Genome Center (<http://www-shgc.stanford.edu/>). Maps were integrated manually to identify markers mapping to the disorder region. A list of the markers is provided in Table 2.

5           2.       Marker Development. Sequences for existing STSs were obtained from the GDB, RHDB (<http://www.ebi.ac.uk/RHdb/>), or NCBI and were used to pick primer pairs (overgos, See Table 2) for BAC library screening. Novel markers were developed either from publicly available genomic sequences, proprietary cDNA sequences or from sequences derived from BAC insert ends (described below). Primers were chosen using a  
10 script that automatically performs vector and repetitive sequence masking using Crossmatch (P. Green, U. of Washington); subsequent primer picking was performed using a customized Filemaker Pro database. Primers for use in PCR-based clone confirmation or radiation hybrid mapping (described below) were chosen using the program Primer3 (Steve Rozen, Helen J. Skaletsky (1996, 1997); Primer3 is available at  
15 [http://www-genome.wi.mit.edu/genome\\_software/other/primer3.html](http://www-genome.wi.mit.edu/genome_software/other/primer3.html)).

Table 2:

Overgo	Locu s	DNA Type	Gene	Forward Primer	Reverse Primer
B0702C13A1x		BACend		GTAAGTAACAGAAATGGACTTTGA	GAGAGGAACAGCATCAAAGTC
A005Q05		EST		CAAACAGGGTCCACCGTGGAAA	GTGTTTCAGCCACATTTCACG
Th		Gene	Mucin 8 (MUC8)	ATCCACCGCTAGAAACCCACTC	GACCATCAACTGATGAGTGGGT
B0702C13A1y		BACend		TCATGGGGGTGCTTTGACCTTG	TGGCCTCAAAGGCTCAAGGTCA

3.       Radiation Hybrid (RH) Mapping. Radiation hybrid mapping was performed  
20 against the Genebridge4 panel (Gyapay, et al., (1996), *Hum. Mol. Genet.* 5:339-46) purchased from Research Genetics, in order to refine the chromosomal localization of genetic markers used in genotyping as well as to identify, confirm and refine localizations of markers from proprietary sequences. Standard PCR procedures were used for typing the RH panel with markers of interest. Briefly, 10 µl PCR reactions contained 25 ng  
25 DNA of each of the 93 Genebridge4 RH samples. PCR products were electrophoresed in 2% agarose gels (Sigma) containing 0.5 µg/ml ethidium bromide in 1X TBE at 150 volts for 45 min. The electrophoresis units used were the Model A3-1 systems from Owl

5 Scientific Products. Typically, gels contained 10 tiers of lanes with 50 wells/tier. Molecular weight markers (100 bp ladder, GIBCO/BRL) were loaded at both ends of the gel. Images of the gels were captured with a Kodak DC40 CCD camera and processed with Kodak 1D software. The gel data were exported as tab delimited text files; names of the files included information about the panel screened, the gel image files and the  
10 marker screened. These data were automatically imported using a customized Perl script into Filemaker databases for data storage and analysis. The data were then automatically formatted and submitted to an internal server for linkage analysis to create a radiation hybrid map using RHMAPPER (Stein, L., Kruglyak, L., Slonim, D., and El Lander (1995); available from the Whitehead Institute/MIT Center for Genome Research, at  
15 <http://www.genome.wi.mit.edu/ftp/pub/software/rhmapper/>, and via anonymous ftp to <ftp.genome.wi.mit.edu>, in the directory /pub/software/rhmapper.) The RH mapping results obtained for Gene 214 indicate that it is present in the 12Q 12-qter region at 507.12 cRays on the 684 coordinate system.

4. BAC Library Screening. The protocol used for BAC library screening was  
20 based on the "overgo" method, originally developed by John McPherson at Washington University in St. Louis (<http://www.tree.caltech.edu/protocols/overgo.html>, and Cai, W-W., et al., (1998), *Genomics* 54:387-397). This method involves filling in the overhangs generated after annealing two primers, each 22 nucleotides in length, that overlap by 8 nucleotides. The resulting labeled 36 bp product is then used in hybridization-based  
25 screening of high density grids derived from the RPCI-11 BAC library (Pieter deJong, Roswell Park Cancer Institute, <http://bacpac.med.buffalo.edu>). Typically, 15 probes were pooled together in one hybridization of 12 filters (13.5 genome equivalents).

Stock solutions (2  $\mu$ M) of combined complementary oligos were heated at 80°C for 5 min, then placed at 37°C for 10 min followed by storage on ice. Labeling reactions  
30 were set up as follows: 1.0  $\mu$ l H<sub>2</sub>O, 5  $\mu$ l mixed oligos – 2  $\mu$ M each, 0.5  $\mu$ l BSA (2 mg/ml), 2  $\mu$ l OLB(-A, -C, -N6) Solution (see below), 0.5  $\mu$ l <sup>32</sup>P-dATP (3000 Ci/mmol),

5 0.5  $\mu$ l  $^{32}$ P-dCTP (3000 Ci/mmol), 0.5  $\mu$ l Klenow fragment (5U/ $\mu$ l). The reaction was incubated at room temperature for 1 hr followed by removal of unincorporated nucleotides with Sephadex G50 spin columns.

OLB(-A, -C, -N6) Solution

Solution O - 1.25 M Tris-HCL, pH 8, 125 M  $MgCl_2$

10 Solution A - 1ml Solution O, 18 $\mu$ l 2-mercaptoethanol, 5 $\mu$ l 0.1M dTTP, 5 $\mu$ l 0.1M dGTP  
Solution B - 2M HEPES-NaOH, pH 6.6

Solution C - 3mM Tris-HCl, pH 7.4, 0.2mM EDTA

Solutions A, B, and C were combined to a final ratio of 1:2.5:1.5, aliquots were stored at -20°C.

15 High density BAC library membranes were pre-wetted in 2X SSC at 58°C.  
Filters were then drained slightly and placed in hybridization solution (1% Bovine serum albumin, 1 mM EDTA - pH 8.0, 7% SDS, and 0.5 M sodium phosphate) pre-warmed to 58°C and incubated at 58°C for 2-4 hr. Typically, 6 filters were hybridized per container.  
Ten ml of pre-hybridization solution were removed, combined with the denatured overgo  
20 probes, and added back to the filters. Hybridization was performed overnight at 58°C.  
The hybridization solution was removed and filters were washed once in 2X SSC, 0.1% SDS, followed by a 30 minute wash in the same solution but at 58°C. Filters were then washed in 1.5X SSC, 0.1% SDS at 58°C for 30 min. 0.5X SSC, 0.1%SDS at 58°C for 30 min and finally in 0.1X SSC, 0.1% SDS at 58°C for 30 min. Filters were then wrapped in  
25 Saran Wrap and exposed to film overnight. To remove bound probe, filters were treated in 0.1X SSC, 0.1% SDS pre-warmed to 95°C and allowed to return to room temperature.  
Clone addresses were determined as described by instructions supplied by RPCI.

Recovery of clonal BAC cultures from the library involved streaking out a sample from the appropriate library well onto LB agar (Maniatis, T., Fritsch, E. F., and J.  
30 Sambrook, (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) containing 12.5  $\mu$ g/ml chloramphenicol (Sigma)

5 and incubating overnight. A single colony and a portion of the initial streak quadrant were inoculated into 400 µl LB plus chloramphenicol in wells of a 96 well plate. Cultures were grown overnight at 37°C. For storage, 100 µl of 80% glycerol was added and the plates placed at -80°C. To determine the marker content of clones, aliquots of the 96 well plate cultures were transferred to the surface of nylon filters (GeneScreen Plus,  
10 NEN) placed on LB/chloramphenicol Petri plates. Colonies were grown overnight at 37°C and colony lysis was performed as follows: Filters were placed on pools of 10% SDS for 3 min, 0.5 N NaOH, 1.5 M NaCl for 5 min, and 0.5 M Tris-HCl, pH 7.5, 1 M NaCl for 5 min. Filters were then air dried and washed free of debris in 2X SSC for 1 hr. The filters were air dried for at least 1 hr and DNA crosslinked linked to the membrane  
15 using standard conditions. Probe hybridization and filter washing were performed as described above for the primary library screening. Confirmed clones were stored in LB containing 15% glycerol.

In some cases polymerase chain reaction (PCR) was used to confirm the marker content of clones. PCR conditions for each primer pair were initially optimized with  
20 respect to MgCl<sub>2</sub> concentration. The standard buffer was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.2 µM each primer, 2.7 ng/µl human DNA, 0.25 units of AmpliTaq (Perkin Elmer) and MgCl<sub>2</sub> concentrations of 1.0 mM, 1.5 mM, 2.0 mM or 2.4 mM. Cycling conditions included an initial denaturation at 94°C for 2 minutes followed by 40 cycles at 94°C for 15 seconds, 55°C for 25 seconds, and 72°C for  
25 25 seconds followed by a final extension at 72°C for 3 minutes. Depending on the results from the initial round of optimization the conditions were further optimized if necessary. Variables included increasing the annealing temperature to 58°C or 60°C, increasing the cycle number to 42 and the annealing and extension times to 30 seconds, and using AmpliTaqGold (Perkin Elmer).

30 5. BAC DNA Preparation. Several different types of DNA preparation methods were used for isolation of BAC DNA. The manual alkaline lysis miniprep

5 protocol listed below (Maniatis, T., Fritsch, E. F., and J. Sambrook, (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) was successfully used for most applications, i.e., restriction mapping, CHEF gel analysis and FISH mapping, but was not reproducibly successful in endsequencing. The Autogen protocol described below was used specifically for BAC DNA preparation for  
10 endsequencing purposes.

For manual alkaline lysis BAC minipreps, bacteria were grown in 15 ml Terrific Broth containing 12.5 µg/ml chloramphenicol in a 50 ml conical tube at 37°C for 20 hrs with shaking at 300 rpm. The cultures were centrifuged in a Sorvall RT 6000 D at 3000 rpm (1800xg) at 4°C for 15 min. The supernatant was then aspirated as completely as  
15 possible. In some cases cell pellets were frozen at -20°C at this step for up to 2 weeks. The pellet was then vortexed to homogenize the cells and minimize clumping. 250 µl of P1 solution (50 mM glucose, 15 mM Tris-HCl, pH 8, 10 mM EDTA, and 100µg/ml RNase A) was added and the mixture pipeted up and down to mix. The mixture was then transferred to a 2 ml Eppendorf tube. 350 µl of P2 solution (0.2 N NaOH, 1% SDS) was  
20 then added, and the mixture mixed gently and incubated for 5 min at room temperature. 350 µl of P3 solution (3M KOAc, pH 5.5) was added and the mixture mixed gently until a white precipitate formed. The solution was incubated on ice for 5 min and then centrifuged at 4°C in a microfuge for 10 min. The supernatant was transferred carefully (avoiding the white precipitate) to a fresh 2 ml Eppendorf tube, and 0.9 ml of isopropanol  
25 was added; the solution was mixed and left on ice for 5 min. The samples were centrifuged for 10 min, and the supernatant removed carefully. Pellets were washed in 70% ethanol and air dried for 5 min. Pellets were resuspended in 200 µl of TE8 (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, pH 8.0), and RNase (Boehringer Mannheim) added to 100 µg/ml. Samples were incubated at 37°C for 30 min, then precipitated by addition of  
30 NH<sub>4</sub>OAc to 0.5 M and 2 volumes of ethanol. Samples were centrifuged for 10 min, and the pellets washed with 70% ethanol followed by air drying and dissolving in 50 µl TE8.

5 Typical yields for this DNA prep were 3-5 µg/ 15 ml bacterial culture. Ten to 15 µl were used for *EcoRI* restriction analysis; 5 µl was used for NotI digestion and clone insert sizing by CHEF gel electrophoresis.

Autogen 740 BAC DNA preparations for endsequencing were prepared by dispensing 3 ml of LB media containing 12.5 µg/ml of chloramphenicol into autoclaved  
10 Autogen tubes. A single tube was used for each clone. For inoculation, glycerol stocks were removed from -70°C storage and placed on dry ice. A small portion of the glycerol stock was removed from the original tube with a sterile toothpick and transferred into the Autogen tube; the toothpick was left in the Autogen tube for at least two minutes before discarding. After inoculation the tubes were covered with tape making sure the seal was  
15 tight. When all samples were inoculated, the tube units were transferred into an Autogen rack holder and placed into a rotary shaker at 37°C for 16-17 hours at 250 rpm. Following growth, standard conditions for BAC DNA preparation, as defined by the manufacturer, were used to program the Autogen. Samples were not dissolved in TE8 as part of the program - DNA pellets were left dry. When the program was complete the  
20 tubes were removed from the output tray and 30 µl of sterile distilled and deionized H<sub>2</sub>O was added directly to the bottom of the tube. The tubes were then gently shaken for 2-5 seconds and then covered with parafilm and incubated at room temperature for 1-3 hours. DNA samples were then transferred to an Eppendorf tube and used either directly for sequencing or stored at 4°C for later use.

25 6. BAC Clone Characterization. DNA samples prepared either by manual alkaline lysis or the Autogen protocol were digested with *EcoRI* for analysis of restriction fragment sizes. These data were used to compare the extent of overlap among clones. Typically 1-2 µg were used for each reaction. Reaction mixtures included: 1X Buffer 2 (New England Biolabs), 0.1 mg/ml bovine serum albumin (New England Biolabs), 50  
30 µg/ml RNase A (Boehringer Mannheim), and 20 units of *EcoRI* (New England Biolabs) in a final volume of 25 µl. Digestions were incubated at 37°C for 4-6 hours. BAC DNA

5 was also digested with NotI for estimation of insert size by CHEF gel analysis (see below). Reaction conditions were identical to those for *EcoRI* except that 20 units of NotI were used. Six µl of 6X Ficoll loading buffer containing bromphenol blue and xylene cyanol was added prior to electrophoresis.

*EcoRI* digests were analyzed on 0.6% agarose (Seakem, FMC Bioproducts) in 1X  
10 TBE containing 0.5 µg/ml ethidium bromide. Gels (20cm X 25 cm) were electrophoresed in a Model A4 electrophoresis unit (Owl Scientific) at 50 volts for 20-24 hrs. Molecular weight size markers included undigested lambda DNA, HindIII digested lambda DNA, and HaeIII digested .X174 DNA. Molecular weight markers were heated at 65°C for 2 min prior to loading the gel. Images were captured with a Kodak DC40  
15 CCD camera and analyzed with Kodak 1D software.

NotI digests were analyzed on a CHEF DRII (BioRad) electrophoresis unit according to the manufacturer's recommendations. Briefly, 1% agarose gels (BioRad pulsed field grade) were prepared in 0.5X TBE, equilibrated for 30 min in the electrophoresis unit at 14 °C, and electrophoresed at 6 volts/cm for 14 hrs with  
20 circulation. Switching times were ramped from 10 sec to 20 sec. Gels were stained after electrophoresis in 0.5 µg/ml ethidium bromide. Molecular weight markers included undigested lambda DNA, HindIII digested lambda DNA, lambda ladder PFG ladder, and low range PFG marker (all from New England Biolabs).

7. BAC Endsequencing. The sequence of BAC insert ends utilized DNA  
25 prepared by either of the two methods described above. The ends of BAC clones were sequenced for the purpose of filling gaps in the physical map and for gene discovery information. The following vector primers specific to the BAC vector pBACe3.6 were used to generate endsequence from BAC clones:

pBAC 5'-2 TGT AGG ACT ATA TTG CTC

30 pBAC 3'-1 CGA CAT TTA GGT GAC ACT

5           The following sequencing protocol using ABI dye-terminator chemistry was used to set up sequencing reactions for 96 clones. The BigDye (Mix: Perkin Elmer/ABI BigDye) Terminator Ready Reaction Mix with AmpliTaq<sup>®</sup> FS, Part number 4303151, was used for sequencing with fluorescently labelled dideoxy nucleotides. A master sequencing mix was prepared for each primer reaction set including:

- 10   1600 µl of BigDye terminator mix (ABI)  
800 µl of 5X CSA buffer (ABI)  
800 µl of primer (either pBAC 5'-2 or pBAC 3'-1 at 3.2 µM)

          The sequencing cocktail was vortexed to ensure it was well-mixed and 32 µl was aliquoted into each PCR tube. Eight µl of the Autogen DNA for each clone was  
15   transferred from the DNA source plate to a corresponding well of the PCR plate. The PCR plates were sealed tightly and centrifuged briefly to collect all the reagents. Cycling conditions were as follows:

- 95°C for 5 minutes  
          95°C for 30 seconds  
20       50°C for 20 seconds  
          65°C for 4 minutes  
          Go to steps 2 through 4 above for an additional 74 times  
          4°C forever

          At the end of the sequencing reaction, the plates were removed from the  
25   thermocycler and centrifuged briefly. Centri•Sep 96 plates were then used according to manufacturer's recommendation to remove unincorporated nucleotides, salts and excess primers. Each sample was resuspended in 1.5 µl of loading dye of which 1.3 µl was loaded on ABI 377 Fluorescent Sequencers. The resulting endsequences were then used to develop markers to rescreen the BAC library for filling gaps and were also analyzed by  
30   BLAST searching for EST or gene content.

5    **E.      SUB-CLONING AND SEQUENCING OF BACS FROM 12q23-qter**

The physical map of the chromosome 12 region provides the BAC clone and location for use as sequencing templates (see Figure 2). DNA sequencing of the BAC RPCI-11\_0702C13 from the region is contained within (SEQ ID NO: 1).

10        DNA for BAC RPCI-11\_0702C13 (the "BAC DNA") was isolated according to one of two protocols: either a Qiagen purification of BAC DNA (Qiagen, Inc., Chatsworth, California, per manufacturer's instructions) or a manual purification using a method which is a modification of the standard alkaline lysis/Cesium Chloride preparation of plasmid DNA (see e.g., Ausubel et al, (1997), Current Protocols in Molecular Biology, John Wiley & Sons). Briefly, for the manual protocol, cells were  
15    pelleted, resuspended in GTE (50 mM glucose, 25 mM Tris-Cl (pH 8), 10 mM EDTA) and lysozyme (50 mg/ml solution), followed by NaOH/SDS (1% SDS/.2N NaOH) and then an ice-cold solution of 3M KOAc (pH 4.5-4.8). RNaseA was added to the filtered supernatant, followed by treatment with Proteinase K and 20% SDS. The DNA was then precipitated with isopropanol, dried and resuspended in TE (10 mM Tris, 1 mM EDTA  
20    (pH 8.0)). The BAC DNA was further purified by Cesium Chloride density gradient centrifugation (Ausubel et al, (1997), Current Protocols in Molecular Biology, John Wiley & Sons).

Following isolation, the BAC DNA was hydrodynamically sheared using HPLC (Hengen, et al., (1997), *Trends in Biochem. Sci.*, 22:273-274) to an insert size of 2000-  
25    3000 bp. After shearing, the DNA was concentrated and separated on a standard 1% agarose gel. A single fraction, corresponding to the approximate size, was excised from the gel and purified by electroelution (Sambrook et al, (1989), *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring, NY).

The purified DNA fragments were then blunt-ended using T4 DNA polymerase.  
30    The healed DNA was then ligated to unique BstXI-linker adapters (5' GTCTTCACCACGGGG and 5' GTGGTGAAGAC in 100-1000 fold molar excess).

5 These linkers are complimentary to the BstXI-cut pMPX vectors, while the overhang is not self-complimentary. Therefore, the linkers will not concatemerize nor will the cut-vector re-ligate to itself easily. The linker-adapted inserts were separated from unincorporated linkers on a 1% agarose gel and purified using GeneClean (BIO 101, Inc.). The linker-adapted insert was then ligated to a modified pBlueScript vector to  
10 construct a "shotgun" subclone library. The vector contains an out-of-frame lacZ gene at the cloning site which becomes in-frame in the event that an adapter-dimer is cloned, allowing these to be avoided by their blue color.

All subsequent steps were based on sequencing by ABI377 automated DNA sequencing methods. Only major modifications to the protocols are highlighted. Briefly,  
15 the library was then transformed into DH5-competent cells (Gibco/BRL, DH5-transformation protocol). Quality was assessed by plating onto antibiotic plates containing ampicillin and IPTG/Xgal. The plates were incubated overnight at 37°C. Successful transformants were then used for plating of clones and picking for sequencing. The cultures were grown overnight at 37°C. DNA was purified using a silica bead DNA  
20 preparation (Ng et al, *Nucl. Acids Res.*, 24:5045-5047 (1996)) method. In this manner, 25 µg of DNA was obtained per clone.

These purified DNA samples were then sequenced using ABI dye-terminator chemistry. The ABI dye terminator sequence reads were run on ABI377 machines and the data were directly transferred to UNIX machines following lane tracking of the gels. All  
25 reads were assembled using PHRAP (P. Green, Abstracts of DOE Human Genome Program Contractor-Grantee Workshop V, Jan. 1996, p.157) with default parameters and quality scores. SEQ ID NO:1 comprises a portion of the BAC which includes the genomic sequence of Gene 214

30 **F. GENE IDENTIFICATION**

5 Any gene or EST mapping to the interval based on public map data or proprietary map data was considered a candidate disorder gene.

1. Gene Identification from clustered DNA fragments. DNA sequences corresponding to gene fragments in public databases (Genbank and human dbEST) and proprietary cDNA sequences (IMAGE consortium and direct selected cDNAs) were  
10 masked for repetitive sequences and clustered using the PANGEA Systems (Oakland, CA) EST clustering tool. The clustered sequences were then subjected to computational analysis to identify regions bearing similarity to known genes. This protocol included the following steps:

i. The clustered sequences were compared to the publicly available  
15 Unigene database (National Center for Biotechnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; www.ncbi.nlm.nih.gov) using the blastn2 algorithm (Altschul et al, *Nucl. Acids Res.*, 25:3389-3402 (1997)). The parameters for this search were: E=0.05, v=50, B=50 (where E is the expected probability score cutoff, V is the number of database entries returned in  
20 the reporting of the results, and B is the number of sequence alignments returned in the reporting of the results (Altschul et al, *J. Mol. Biol.*, 215:403-410 (1990)).

ii. The clustered sequences were compared to the Genbank database (National Center for Biotechnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; www.ncbi.nlm.nih.gov) using  
25 blastn2 (Altschul et al, *Nucl. Acids. Res.*, 25:3389-3402 (1997)). The parameters for this search were E=0.05, V=50, B= 50, where E, V, and B are defined as above.

iii. The clustered sequences were translated into protein for all six reading frames, and the protein sequences were compared to a non-redundant protein database compiled from Genpept Swissprot PIR (National Center for Biotchnology Information,  
30 National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894;

5 www.ncbi.nlm.nih.gov). The parameters for this search were  $E=0.05$ ,  $V=50$ ,  $B=50$ , where  $E$ ,  $V$ , and  $B$  are defined as above.

iv. The clustered sequences were compared to BAC sequences (see below) using blastn2 (Altschul et al, *Nucl. Acids. Res.*, 25:3389-3402 (1997)). The parameters for this search were  $E=0.05$ ,  $V=50$ ,  $B=50$ , where  $E$ ,  $V$ , and  $B$  are defined as  
10 above.

2. Gene Identification from BAC Genomic Sequence. Following assembly of the BAC sequences into contigs, the contigs were subjected to computational analyses to identify coding regions and regions bearing DNA sequence similarity to known genes. This protocol included the following steps:

15 i. Contigs were degapped. The sequence contigs often contain symbols (denoted by a period symbol) that represent locations where the individual ABI sequence reads have insertions or deletions. Prior to automated computational analysis of the contigs, the periods were removed. The original data were maintained for future reference.

20 ii. BAC vector sequences were "masked" within the sequence by using the program crossmatch (Phil Green, <http://chimera.biotech.washington.edu/UWGC>). Since the shotgun library construction detailed above left some BAC vector in the shotgun libraries, this program was used to compare the sequence of the BAC contigs to the BAC vector and to mask any vector sequence prior to subsequent steps. Masked  
25 sequence was marked by an "X" in the sequence files, and remained inert during subsequent analyses.

iii. *E. coli* sequences contaminating the BAC sequences were masked by comparing the BAC contigs to the entire *E. coli* DNA sequence.

30 iv. Repetitive elements known to be common in the human genome were masked using crossmatch. In this implementation of crossmatch, the BAC sequence is compared to a database of human repetitive elements (Jerzy Jerka, Genetic Information

5 Research Institute, Palo Alto, CA). The masked repeats were marked by X and remained inert during subsequent analyses.

v. The location of exons within the sequence was predicted using the MZEF computer program (Zhang, *Proc. Natl. Acad. Sci.*, 94:565-568 (1997); GenScan(Burge and Karlin, *J. Mol. Biol.*, 268:78-94)).

10 vi. The sequence was compared to the publicly available unigene database (National Center for Biotechnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using the blastn2 algorithm (Altschul et al, *Nucl. Acids Res.*, 25:3389-3402 (1997)). The parameters for this search were: E=0.05, v=50, B=50 (where E is the expected probability score cutoff,  
15 V is the number of database entries returned in the reporting of the results, and B is the number of sequence alignments returned in the reporting of the results (Altschul et al, *J. Mol. Biol.*, 215:403-410 (1990)).

vii. The sequence was translated into protein for all six reading frames, and the protein sequences were compared to a non-redundant protein database compiled  
20 from Genpept Swissprot PIR (National Center for Biotchnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The parameters for this search were E=0.05, V=50, B= 50, where E, V, and B are defined as above.

viii. The BAC DNA sequence was compared to a database of clustered  
25 sequences using blastn2 (Altschul et al, *Nucl. Acids. Res.*, 25:3389-3402 (1997)). The parameters for this search were E=0.05, V=50, B=50, where E, V, and B are defined as above. The database of clustered sequences was prepared utilizing a proprietary clustering technology (Pangea Systems, Inc.) using cDNA clones derived from direct selection experiments (described below), human dbEST mapping to the 12q23-qter  
30 region, proprietary cDNAs, Genbank genes and IMAGE consortium cDNA clones.

5           ix. The BAC sequence was compared to the sequences derived from the ends of BACs from the region on chromosome 12 using blastn2 (Altschul et al, *Nucl. Acids. Res.*, 25:3389-3402 (1997)). The parameters for this search were  $E=0.05$ ,  $V=50$ ,  $B=50$ , where  $E$ ,  $V$ , and  $B$  are defined as above.

10           x. The BAC sequence was compared to the Genbank database (National Center for Biotechnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using blastn2 (Altschul et al, *Nucl. Acids. Res.*, 25:3389-3402 (1997)). The parameters for this search were  $E=0.05$ ,  $V=50$ ,  $B=50$ , where  $E$ ,  $V$ , and  $B$  are defined as above.

15           xi. The BAC sequence was compared to the STS division of Genbank database (National Center for Biotechnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using blastn2 (Altschul et al., 1997). The parameters for this search were  $E=0.05$ ,  $V=50$ ,  $B=50$ , where  $E$ ,  $V$ , and  $B$  are defined as above.

20           xii. The BAC sequence was compared to the Expressed Sequence Tag (EST) Genbank database (National Center for Biotchnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using blastn2 (Altschul et al., *Nucl. Acids. Res.*, 25:3389-3402 (1997)). The parameters for this search were  $E=0.05$ ,  $V=50$ ,  $B=50$ , where  $E$ ,  $V$ , and  $B$  are defined as above.

25           xiii. The BAC sequence was compared to the Expressed Sequence Tag (EST) Genbank database (National Center for Biotchnology Information, National Library of Medicine, 38A, 8N905, 8600 Rockville Pike, Bethesda, MD 20894; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using blastn2 (Altschul et al., *Nucl. Acids. Res.*, 25:3389-3402 (1997)). The parameters for this search were  $E=0.05$ ,  $V=50$ ,  $B=50$ , where  $E$ ,  $V$ , and  $B$  are  
30           defined as above.

#### G. cDNA CLONING AND EXPRESSION ANALYSIS

5           1.       Construction of cDNA libraries. Directionally cloned cDNA libraries  
from normal lung and bronchial epithelium were constructed using standard methods  
described previously (Soares et. al., 1994, Automated DNA Sequencing and Analysis,  
Adams, Fields and Venter, Eds., Academic Press, NY, pages 110-114). Total and  
cytoplasmic RNAs were extracted from tissue or cells by homogenizing the sample in the  
10   presence of Guanidinium Thiocyanate-Phenol-Chloroform extraction buffer (e.g.  
Chomczynski and Sacchi, Anal. Biochem., 162:156-159 (1987)) using a polytron  
homogenizer (Brinkman Instruments). PolyA<sup>+</sup> RNA was isolated from total/cytoplasmic  
RNA using dynabeads-dT according to the manufacturer's recommendations (Dynal,  
Inc.). The ds cDNA synthesized was then ligated into the plasmid vector pBluescript II  
15   KS<sup>+</sup> (Stratagene, La Jolla, California), and the ligation mixture was transformed into E.  
coli host DH10B or DH12S by electroporation (Soares, 1994). Following overnight  
growth at 37°C, DNA was recovered from the E. coli colonies after scraping the plates by  
processing as directed for the Mega-prep kit (Qiagen, Chatsworth, California). The  
quality of the cDNA libraries was estimated by counting a portion of the total number of  
20   primary transformants, determining the average insert size and the percentage of plasmids  
with no cDNA insert. Additional cDNA libraries (human total brain, heart, kidney,  
leukocyte, and fetal brain) were purchased from Life Technologies, Bethesda, Maryland.

cDNA libraries, both oligo (dT) and random hexamer-primed were used for  
isolating cDNA clones mapping within the disorder critical region. Four 10 x 10 arrays of  
25   each of the cDNA libraries were prepared as follows: the cDNA libraries were titrated to  
2.5 x 10<sup>6</sup> using primary transformants. The appropriate volume of frozen stock was used  
to inoculate 2 L of LB/ampicillin (100 µg/µl). 400 aliquots containing 4 ml of the  
inoculated liquid culture were generated. Each tube contained about 5000 cfu. The tubes  
were incubated at 30°C overnight with shaking until an OD of 0.7-0.9 was obtained.  
30   Frozen stocks were prepared for each of the cultures by aliquotting 300 µl of culture and  
100 µl of 80% glycerol. Stocks were frozen in a dry ice/ethanol bath and stored at -70°C.

5 DNA was isolated from the remaining culture using the Qiagen (Chatsworth, CA) spin mini-prep it according to the manufacturer's instructions. The DNAs from the 400 cultures were pooled to make 80 column and row pools. Markers were designed to amplify putative exons from candidate genes. Once a standard PCR condition was identified and specific cDNA libraries were determined to contain cDNA clones of interest, the markers were used to screen the arrayed library. Positive addresses indicating the presence of cDNA clones were confirmed by a second PCR using the same markers.

Once a cDNA library was identified as likely to contain cDNA clones corresponding to a specific transcript of Gene 214, it was used to isolate a clone or clones containing cDNA inserts. This was accomplished by a modification of the standard "colony screening" method (Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY (1989)). Specifically, twenty 150 mm LB+ampicillin agar plates were spread with 20,000 colony forming units (cfu) of cDNA library and the colonies allowed to grow overnight at 37°C. Colonies were transferred to nylon filters (Hybond from Amersham, or equivalent) and duplicates prepared by pressing two filters together essentially as described (Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY (1989)). The "master" plate was then incubated an additional 6-8 hrs to allow the colonies additional growth. The DNA from the bacterial colonies was then bound onto the nylon filters by treating the filters sequentially with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for two minutes, neutralization solution (0.5 M Tris-Cl pH 8.0, 1.5 M NaCl) for two minutes (twice). The bacterial colonies were removed from the filters by washing in a solution of 2X SSC/ 2% SDS for one minute while rubbing with tissue paper. The filters were air dried and baked under vacuum at 80°C for 1-2 hrs to cross link the DNA to the filters.

5 cDNA hybridization probes were prepared by random hexamer labelling  
(Fineberg and Vogelstein, Anal. Biochem., 132:6-13 (1983)) or by including gene-  
specific primers and no random hexamers in the reaction (for small fragments). The  
colony membranes were then pre-washed in 10 mM Tris-Cl pH 8.0, 1 M NaCl, 1 mM  
EDTA, 0.1% SDS for 30 minutes at 55°C. Following the pre-wash, the filters were pre-  
10 hybridized in > 2 ml/filter of 6X SSC, 50 % deionized formamide, 2% SDS, 5X  
Denhardt's solution, and 100 mg/ml denatured salmon sperm DNA, at 42°C for 30  
minutes. The filters were then transferred to hybridization solution (6X SSC, 2% SDS,  
5X Denhardt's, 100 mg/ml denatured salmon sperm DNA) containing denatured  $\alpha$ -<sup>32</sup>P-  
dCTP-labelled cDNA probe and incubated overnight at 42°C.

15 The following morning, the filters were washed under constant agitation in 2X  
SSC, 2% SDS at room temperature for 20 minutes, followed by two washes at 65°C for  
15 minutes each. A second wash was performed in 0.5 X SSC, 0.5% SDS for 15 minutes  
at 65°C. Filters were then wrapped in plastic wrap and exposed to radiographic film.  
Individual colonies on plates were aligned with the autoradiograph and positive clones  
20 picked into a 1 ml solution of LB Broth containing ampicillin. After shaking at 37°C for  
1-2 hours, aliquots of the solution were plated on 150 mm plates for secondary screening.  
Secondary screening was identical to primary screening (above) except that it was  
performed on plates containing ~250 colonies so that individual colonies could be clearly  
identified. Positive cDNA clones were characterized by restriction endonuclease  
25 cleavage, PCR, and direct sequencing to confirm the sequence identity between the  
original probe and the isolated clone.

To obtain the full-length cDNA, novel sequence from the 5'-end of the clone was  
used to reprobe the library. This process is repeated until the length of the cDNA cloned  
matched that of the mRNA, estimated by Northern analysis.

30 Rapid Amplification of cDNA ends (RACE) was performed following the  
manufacturer's instructions using a Marathon cDNA Amplification Kit (Clontech, Palo

5 Alto, CA) as a method for cloning the 5' and 3' ends of candidate genes. cDNA pools were prepared from total RNA by performing first strand synthesis, where a sample of total RNA sample was mixed with a modified oligo (dT) primer, heated to 70°C, cooled on ice and followed by the addition of: 5X first strand buffer, 10 mM dNTP mix, and AMV Reverse Transcriptase (20 U/μl). The reaction mixture was incubated at 42°C for  
10 an hour and placed on ice. For second strand synthesis, the following components were added directly to the reaction tube: 5X second strand buffer, 10 mM dNTP mix, sterile water, 20X second strand enzyme cocktail and the reaction tube was incubated at 16°C for 1.5 hours. T4 DNA Polymerase was added to the reaction tube and incubated at 16°C for 45 minutes. The second-strand synthesis was terminated with the addition of an  
15 EDTA/Glycogen mix. The sample was subjected to a phenol/chloroform extraction and an ammonium acetate precipitation. The cDNA pools were checked for quality by analyzing on an agarose gel for size distribution. Marathon cDNA adapters were then ligated onto the cDNA ends. The specific adapters contained priming sites that allowed for amplification of either 5' or 3' ends, and varied depending on the orientation of the  
20 gene specific primer (GSP) that was chosen. An aliquot of the double stranded cDNA was added to the following reagents: 10 μM Marathon cDNA adapter, 5X DNA ligation buffer, T4 DNA ligase. The reaction was incubated at 16°C overnight and heat inactivated to terminate the reaction. PCR was performed by the addition of the following to the diluted double stranded cDNA pool: 10X cDNA PCR reaction buffer, 10  
25 μM dNTP mix, 10 μM GSP, 10 μM AP1 primer (kit), 50X Advantage cDNA Polymerase Mix. Thermal Cycling conditions were 94°C for 30 seconds, 5 cycles of 94°C for 5 seconds, 72°C for 4 minutes, 5 cycles of 94°C for 5 seconds, 70°C for 4 minutes, 23 cycles of 94°C for 5 seconds, 68°C for 4 minutes. After the first round of PCR was performed using the GSP to extend to the end of the adapter to create the adapter primer  
30 binding site, exponential amplification of the specific cDNA of interest was performed. Usually, a second, nested PCR was performed to provide specificity. The RACE product

5 was analyzed on an agarose gel. Following excision from the gel and purification (GeneClean, BIO 101), the RACE product was then cloned into pCTNR (General Contractor DNA Cloning System, 5' - 3', Inc.) and sequenced to verify that the clone was specific to Gene 214.

2. Expression Analysis. To characterize the expression of Gene 214, a series  
10 of experiments were performed. First, oligonucleotide primers were designed for use in the polymerase chain reaction (PCR) so that portions of a cDNA, EST, or genomic DNA could be amplified from a pool of DNA molecules or RNA population (RT-PCR). The PCR primers were used in a reaction containing genomic DNA to verify that they generated a product of the predicted size (based on the genomic sequence). A critical  
15 piece of data that is required when characterizing novel genes is the length, in nucleotides, of the processed transcript or messenger RNA (mRNA). Those skilled in the art primarily determine the length of an mRNA by Northern analysis (Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY (1989)). Probes were generated using one of the methods described below.  
20 Briefly, sequence verified IMAGE consortium cDNA clones were digested with appropriate restriction endonucleases to release the insert. The restriction digest was electrophoresed on an agarose gel and the bands containing the insert were excised. The gel piece containing the DNA insert was placed in a Spin-X (Corning Costar Corporation, Cambridge, MA) or Supelco spin column (Supelco Park, PA) and spun at high speed for  
25 15 mins. The DNA was ethanol precipitated and resuspended in TE. Alternatively, PCR products obtained from genomic DNA or RT-PCR were also purified as described above. Inserts purified from IMAGE clones were random primer labelled (Feinberg and Vogelstein) to generate probes for hybridization. Probes from purified PCR products were generated by incorporation of  $\alpha$ -<sup>32</sup>P-dCTP in second round of PCR. Figure 9 is the  
30 Northern blot for Gene 214 which includes PolyA+ selected RNA from 1) a lymphoblast cell line from an asthmatic individual, 2) lung and 3) trachea. Expression of Gene 214

5 was detected in lung at moderate levels, with a weak signal in trachea. Expression was not found in any other tissues examined. The lung-specific expression of Gene 214 implicates it as a gene involved in lung biology and further validates as a candidate asthma gene.

3. RT-PCR. RT-PCR was used as an alternate method to Northern blotting  
10 to detect mRNAs with low levels of expression. Total RNA from multiple human tissues was purchased from Clontech (Palo Alto, CA) and genomic DNA was removed from the total RNA by DNaseI digestion. The "Superscript" Preamplification System for First strand cDNA synthesis" (Life Technologies, Gaithersburg, MD) was used according to manufacturer's specifications with oligo(dT) or random hexamers to synthesize cDNA  
15 from the DNaseI treated total RNA. Gene specific primers were used to amplify the target cDNAs in a 30 µl PCR reaction containing 0.5 µl of first strand cDNA, 1 µl sense primer (10uM), 1 µl antisense primer (10uM), 3 µl dNTPs ( 2 mM), 1.2 µl MgCl<sub>2</sub> (25 mM), 3 µl 10X PCR buffer and 1 unit of Taq Polymerase (Perkin Elmer). The PCR reaction was initially denatured at 94°C for 4 min, then 30 cycles of denaturation at 94°C  
20 for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. PCR products were analyzed on agarose gels.

#### H. CHARACTERISTICS AND FUNCTION OF GENE 214

BAC RP11-702C13 (196Kb) maps to chromosome 12q24 and contains the STS  
25 marker A005Q05 located approximately 165 cM from the telomere of the p-arm of chromosome 12. Gene 214 maps within a 10,318 kb sequenced contig of the BAC RP11-702C13 (Fig. 2). BLAST analysis against DNA and protein databases indicated that a portion of Gene 214 was 100% homologous to a nucleic acid sequence known as mucin 8 (MUC8). Northern blot analysis of Gene 214 detected a 4.4Kb transcript in lung (Fig. 9).  
30 The MUC8 fragment is 1.4Kb in length (Shanker *et.al.*, *Am J. Respir. Cell Mol. Biol.*, 16:232-241 (1997)). Enclosed herein are an additional four alternatively transcribed

5 variants. (SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and SEQ ID NO:10). The five variants of Gene 214/MUC8 contain a putative open reading frames that vary in size, from 1167bp to 1350bp, and thus encode proteins from 388 to 449 amino acids (SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO: 9, and SEQ ID NO:11).

10 Mucin 8 belongs to a growing family of genes that encode mucins. Currently, there are 9 members of this family, MUC1, MUC2, MUC3, MUC4, MUC5, MUC5B, MUC6 and MUC7, and the fragment MUC8. All but MUC3 and MUC6 are expressed in the upper and/or lower respiratory tract (Zuhdi Alimam *et al.*, *Am. J. Respir. Cell Mol. Biol.*, 22:253-260 [2000]). All the mucins share a common characteristic: tandem repeated amino acid  
15 sequences within the protein core. These repeats are rich in serine, threonine and proline and are heavily glycosylated via O-glycosidic bonds. The tandem repeat units vary in length from as few as 8 to as many as 169 amino acids and are always flanked by non-repeat regions. The MUC8 core protein is unique among the mucins, in that it possesses a degenerate 41bp tandem repeat that encodes 2 types of consensus peptide repeats; three 41bp repeats encode  
20 one peptide sequence while a 2bp deletion in the perfect 41bp repeat disrupts the tandem and generates a second smaller repetitive portion of the protein (Shanker *et.al.*, *Biochem. J.*, 300:295-298 (1994).

The respiratory epithelium is protected by a viscoelastic gel, mucus, that is normally produced at low levels. In a healthy individual, 10mls of sputum are transported to the larynx  
25 and swallowed. In asthmatic individuals, mucus production is increased. This causes airway obstruction due to the sputum being very tenacious, and hence forming viscid plugs that can be difficult to expectorate. The overproduction of mucus in asthmatics has been attributed to the increased numbers of goblets cells, goblet cell hyperplasia (GCH), and enlargement of the sub-mucosal glands. GCH is presumed to be due to a combination of mucus gland  
30 stimulation by neural stimuli and inflammatory mediators. *In situ* hybridization revealed that multiple airway mucin genes account for the total mucin secretion derived from the airway

5 epithelia. Further, immunohistochemical staining of tracheobronchial epithelium with polyclonal antibodies raised to MUC8, indicated that the protein was primarily localized to sub-mucosal glands (Shanker *et.al.*, *Am J. Respir. Cell Mol. Biol.*, 16:232-241 [1997]). Therefore it is likely that the relationship and functional role of Gene 214/MUC8 are involved in the pathophysiology of asthma and other respiratory diseases.

## 10 I. MUTATION ANALYSIS

In order to conduct mutation analysis, the genomic structure for Gene 214 was identified. The precise intron-exon junctions were determined based on the consensus sequences at splice junctions. The exon prediction programs MZEF (Zhang, *Proc. Natl. Acad. Sci.*, 94:565-568 (1997); and GenScan (Burge and Karlin, *J. Mol. Biol.*, 268:78-94) were also utilized to help identify the exons.

A combination of fluorescent single stranded confirmation (SSCP) analysis (ABI) and DNA sequencing was utilized to precisely identify and determine the nature of the variant at the nucleotide level. SSCP analysis was used to screen individual DNA for variants. Briefly, polymerase chain reaction (PCR) was used to generate templates from unrealed asthmatic individuals that showed increased sharing for the 12q23-qter chromosomal region and contributed towards linkage. Non-asthmatic individuals were used as controls. Enzymatic amplification of genes within the asthma region on 12q23-qter was accomplished using PCR with oligonucleotides flanking each exon as well as the putative 5' regulatory elements of each gene. The primers were chosen to amplify each exon as well as 15 or more base pairs within each intron on either side of the splice site. The forward and the reverse primers had two different dye colors to allow analysis of each strand and confirm variants independently. Standard PCR assay was utilized for each exon primer pair following optimization. Buffer and cycling conditions were specific to each primer set. The products were denatured using a formamide dye and

5 electrophoresed on non-denaturing acrylamide gels with varying concentrations of glycerol (at least two different glycerol concentrations).

Primers utilized in fluorescent SSCP experiments to screen coding and non-coding regions of Gene 214 for polymorphisms are provided in Table 3. Column one lists the gene targeted for mutation analysis. Column two lists the specific exon  
10 analyzed. Column three provides the GTC assigned primer name. Columns four and five list the forward primer sequence and reverse primer sequence, respectively.

Table 3:

Gene	Exon	SSCP Assay	Forward Primer	Reverse Primer
214	A	196_214_A_F_197_214_A_R	GCCCTTAGGGAGAGCAGC	CCACATCGTGCCTTTGTGTA
214	B	192_214_B_F_193_214_B_R	CACTGTGTTAAAACGCCTGG	GTTGGGATTACAGGCACGAG
214	B	194_214_B_F_195_214_B_R	CAGAAGCAACCCACATGACC	ACTACAGGTTTGCACCACCA
214	C	626_214_C_F_627_214_C_R	ATGCTCTCCTGATGGCTCCT	AGGGAATGCAGGTGCAAAG
214	C	628_214_C_F_629_214_C_R	ACTCGGGAAAGGAAGGCTCT	CATACCTTGAGTGCACACCG

15 Primers utilized in DNA sequencing for purposes of confirming polymorphisms detected using fluorescent SSCP are provided in Table 4. Column one lists the specific exon sequenced. Column two provides the GTC assigned forward primer name and column three lists the forward primer sequence. Columns four and five lists the GTC  
20 assigned reverse primer name and the corresponding reverse primer sequence, respectively.

Table 4:

Gene	Exon	ForwardPrimer	ForwardSequence	ReversePrimer	ReverseSequence
214	B	MDSeq_15_214_B_F	GACAGTCTGCTCCACATCCA	MDSeq_15_214_B_R	TGGAGATGAAGTCTTGCTCTTG
214	C	MDSeq_110_214_C_F	ATATGTTTGCTGGCTTTGGG	MDSeq_110_214_C_R	CCCAGGCTGTGTGTCCTCTA

25 Single nucleotide polymorphisms (SNPs) that were identified in Gene 214 are provided in Table 5. Column one contains the exon or intron in which the SNP was detected. Column two provides a reference sequence in which the SNP appears

5 underlined. Column three lists the base change of the SNP. Column four details the location of the SNP as intronic or exonic. Column five describes the SNP location of the genomic BAC sequence of SEQ ID NO:1. The SNPs are also described in Figures 8A-8B).

10 Table 5:

Exon	Reference Sequence	PMP	Intron/ Exon	Location
B	ACTACAGGTTTGCACCACCATGTCCTGCTAATTTTTTTT	A>G	Intron	6684
B	TGTGCACTCTTGGGCATACGCCTAGGAGTGGAACTGCTG	C>T	3'UTR	6991
C	GGGCTCTGCGCCACCTCAACCCAGGCGTTTGTTCGCAG	C>T	Intron	3176

Figures 8A-8B illustrate the five different transcripts of Gene 214 and show the genomic structure of the gene. The exons are shown to scale and the SNPs are identified by their location along the genomic BAC DNA (SEQ ID NO:1).

#### J. RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP ASSAY) AND ALLELE SPECIFIC OLIGONUCLEOTIDE ANALYSIS (ASO ASSAY)

20 To identify other individuals with the polymorphisms listed in Table 5, RFLP assay and ASA were performed.

1. RFLP Assay. The amplicon, containing the polymorphism, was PCR amplified using primers that were used to generate a fragment for sequencing (sequencing primers) or SSCP (SSCP primers). The appropriate population of individuals was PCR amplified in 96 well microtitre plates.

Enzymes were purchased from New England Biolabs (NEB). The restriction cocktail containing the appropriate enzyme for the particular polymorphism is added to the PCR product. The reaction is incubated at the appropriate temperature according to the manufacturer's recommendations (NEB) for two to three hours, followed by a 4° C

5 incubation. After digestion, the reactions were size fractionated using the appropriate agarose gel depending on the assay specifications (2.5%, 3%, or metaphor). Gels are electrophoresed in 1X TBE Buffer at 170 Volts for approximately two hours.

The gel is illuminated using ultraviolet light and the image is saved as a Kodak 1D file. Using the Kodak 1D image analysis software, the images are scored and the data  
10 is exported to EXCEL.

2. ASO assay. The amplicon, containing the polymorphism, was PCR amplified using primers that were used to generate a fragment for sequencing (sequencing primers) or SSCP (SSCP primers). The appropriate population of individuals was PCR amplified in 96 well microtitre plates and re-arrayed into 384 well microtitre plates using  
15 a Tecan Genesis RSP200. The amplified products were loaded onto 2% agarose gels and size fractionated at 150V for 5 minutes. The DNA was transferred from the gel to Hybond N+ nylon membrane (Amersham-Pharmacia) using a Vacuum blotter (Bio-Rad). The filter containing the blotted PCR products was transferred to a dish containing 300mls of pre-hybridization solution (5x SSPE {pH7.4}, 2% SDS, 5x Denhardts). The  
20 filter was left in the pre-hybridization solution at 40°C for >1 hour. After pre-hybridization, 10mls of the pre-hybridization solution and the filter were transferred to a washed glass bottle. The allele specific oligonucleotides (ASO) were designed with the polymorphism in the middle. The size of the oligonucleotide was dependent upon the GC content of the sequence around the polymorphism. Those ASOs that had a G or C  
25 polymorphism were designed so that the T<sub>m</sub> was between 54-56°C and those that had an A or T variance were designed so that the T<sub>m</sub> was between 60-64°C. All oligonucleotides were phosphate free at the 5' end and purchased from Gibco BRL. For each polymorphism 2 ASOs were designed: one for each variant.

The two ASOs that represented the polymorphism were resuspended at a  
30 concentration of 1µg/µl and separately end-labeled with γ-ATP<sup>32</sup> (6000Ci/mmol) (NEN) using T4 polynucleotide kinase according to manufacturer recommendations (NEB). The

5 end-labeled products were removed from the unincorporated  $\gamma$ -ATP<sup>32</sup> by passing the reactions through Sephadex G-25 columns according to manufacturers recommendation (Amersham-Pharmacia). The entire end-labeled product of one ASO was added to the bottle containing the appropriate filter and 10mls of hybridization solution. The hybridization reaction was placed in a rotisserie oven (Hybaid) and left at 40°C for a  
10 minimum of 4 hours. The other ASO was stored at -20° C.

After the prerequisite hybridization time had elapsed, the filter was removed from the bottle and transferred to 1 liter of wash solution (0.1x SSPE {pH7.4}, 0.1%SDS) pre-warmed to 45°C. After 15 minutes the filter was transferred to another liter of wash solution (0.1x SSPE {pH7.4}, 0.1%SDS) pre-warmed to 50°C. After 15 minutes the  
15 filter was wrapped in Saran, placed in an autoradiograph cassette and an X-ray film (Kodak) placed on top of the filter. Depending on the efficiency of the end-labeling reaction of the ASO and its hybridization to the filter an image would be observed on the film within an hour. After an image had been captured on film for the 50°C wash, the process was repeated for wash steps at 55°C, 60°C and 65°C. The image that captured the  
20 best result was used.

The ASO was removed from the filter by adding 1 liter of boiling strip solution (0.1x SSPE {pH7.4}, 0.1%SDS). This was repeated two more times. After removing the ASO the filter was pre-hybridized in 300mls of pre-hybridization solution (5x SSPE {pH7.4}, 2% SDS, 5x Denhardts) at 40°C for >1 hour. The second end-labeled ASO  
25 corresponding to the other variant was removed from storage at -20°C and thawed to room temperature. The filter was placed into a glass bottle along with 10mls of hybridization solution and the entire end-labeled product of the second ASO. The hybridization reaction was placed in a rotisserie oven (Hybaid) and left at 40°C for a minimum of 4 hours. After the hybridization, the filter was washed at various  
30 temperatures and images captured on film as described above.

The two films that best captured the allele specific assay with the two ASOs were

5 converted into digital images by scanning them into Adobe PhotoShop. These images were overlaid against each other in Graphic Converter and then scored and stored in FileMaker Pro 4.0.

#### **K. ASSOCIATION STUDY ANALYSIS**

10 In order to determine whether mutations in candidate genes are responsible for the asthma phenotype, association studies are performed using a case-control study design. To avoid issues of population admixture which can bias case-control studies, the unaffected controls were collected in both the US and the UK. A total of three hundred controls were collected, 200 in the UK and 100 in the US. Inclusion into the study  
15 required that the control individual was negative for asthma, as determined by self report of never having asthma, has no first degree relatives with asthma, and was negative for eczema and symptoms indicative of atopy within the past 12 months. Data from an abbreviated questionnaire similar to that administered to the affected sib pair families were collected. Results from skin prick tests to 4 common allergens were also collected.  
20 The results of the skin prick test were used to select a subset of control that were most likely to be asthma and atopy negative.

A subset of unrelated cases are selected from the affected sib pair families based on the evidence for linkage at the chromosomal location of interest. One affected sib from families demonstrating identity-by-descent (IBD) at the appropriate marker loci is  
25 selected. In the selection criteria, preference is given to families with multiple affected sibs all of whom are concordant at the marker locus as well as to families where affected and unaffected sibs are discordant.

For each polymorphism, the frequency of the alleles in the control and case populations is compared using a Fisher exact test. It is expected that a mutation  
30 increasing susceptibility to the disease would be more prevalent in the cases than in the controls, while a protective mutation should be more prevalent in the control group.

Similarly, the genotype frequencies of the SNPs are compared between cases and controls. P-values are computed for both the allele and genotype frequencies. A small p-value is indicative of an association between the SNPs and the disease phenotype. The analysis is repeated for the US and UK population separately, to adjust for the possibility of genetic heterogeneity.

#### 1. Association test with individual SNPs

Statistical analyses for the two SNPs in Gene 214 are presented in Table 8. Column one list the exon containing the SNP of interest. The control ("CNTL") allele frequency and sample size ("N") are in columns two and three. The affected individuals ("CASE") allele frequency and sample size ("N") are listed in columns four and five. The sixth column contains the significance value level of comparison between the control allele frequencies and the case allele frequencies. The SNP in Exon C had allelic frequencies significantly different in the cases versus the controls in the US and combined samples. In the Combined and US population, this SNP was more frequent in the cases (4.1% and 10.4%, respectively) than in the control population (0.8% and 1.3%), and the differences were statistically significance ( $p=0.0099$  and  $p=0.0083$ ). This analysis suggests that Gene 214, is, at least partially responsible for the asthmatic phenotype in those families linked to the chromosome 12 region.

Table 8:

<b>Combined sample</b>						
	Frequencies					ALLELE

EXON	CNTL	N		CASE	N		P- VALUE
B	17.8%	214		20.3%	111		0.4577
C	0.8%	194		4.1%	97		0.0083
<b>US sample</b>							
	Frequencies						ALLELE
EXON	CNTL	N		CASE	N		P- VALUE
B	15.1%	76		16.7%	24		0.8204
C	1.3%	75		10.4%	24		0.0099
<b>UK sample</b>							
	Frequencies						ALLELE
EXON	CNTL	N		CASE	N		P- VALUE
B	19.2%	138		21.3%	87		0.6291
C	0.4%	119		2.1%	73		0.1559

5

## II. PREPARATION OF NUCLEIC ACIDS, VECTORS, TRANSFORMATIONS AND HOST CELLS

10 The nucleic acids of this invention can be produced in large quantities by replication in a suitable host cell. Natural or synthetic nucleic acid fragments, comprising at least ten contiguous bases coding for a desired peptide or polypeptide can be incorporated into recombinant nucleic acid constructs, usually DNA constructs, capable of introduction into and replication in a prokaryotic or eukaryotic cell. Usually the nucleic acid constructs will be suitable for replication in a unicellular host, such as yeast

5 or bacteria, but may also be intended for introduction to (with and without integration within the genome) cultured mammalian or plant or other eukaryotic cells, cell lines, tissues, or organisms. The purification of nucleic acids produced by the methods of the present invention is described, for example, in Sambrook *et al*, *Molecular Cloning. A Laboratory Manual*, 2nd Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY  
10 (1989) or Ausubel *et al*, *Current Protocols in Molecular Biology*, J. Wiley and Sons, NY (1992).

The nucleic acids of the present invention can also be produced by chemical synthesis, *e.g.*, by the phosphoramidite method described by Beaucage *et al*, *Tetra. Letts.*, 22:1859-1862 (1981) or the triester method according to Matteucci, *et al*, *J. Am. Chem.*  
15 *Soc.*, 103:3185 (1981), and can be performed on commercial, automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single-stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

20 These nucleic acids can encode full-length variant forms of proteins as well as the naturally-occurring protein. The variant proteins (which could be especially useful for detection and treatment of disorders) can have the variant amino acid sequences encoded by the polymorphisms described in Table 5, when said polymorphisms are read so as to be in-frame with the full-length coding sequence of which it is a component.

25 Nucleic acid constructs prepared for introduction into a prokaryotic or eukaryotic host will comprise a replication system recognized by the host, including the intended nucleic acid fragment encoding the selected protein or polypeptide, and will preferably also include transcription and translational initiation regulatory sequences operably linked to the protein encoding segment. Expression vectors may include, for example, an origin  
30 of replication or autonomously replicating sequence (ARS) and expression control sequences, a promoter, an enhancer and necessary processing information sites, such as

5 ribosome-binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, and mRNA stabilizing sequences. Secretion signals are also included, where appropriate, whether from a native Gene 214 protein or from other receptors or from secreted proteins of the same or related species, which allow the protein to cross and/or lodge in cell membranes, and thus attain its functional topology, or be  
10 secreted from the cell. Such vectors may be prepared by means of standard recombinant techniques well known in the art and discussed, for example, in Sambrook *et al*, *Molecular Cloning. A Laboratory Manual*, 2nd Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) or Ausubel *et al*, *Current Protocols in Molecular Biology*, J. Wiley and Sons, NY (1992).

15 An appropriate promoter and other necessary vector sequences will be selected so as to be functional in the host, and will include, when appropriate, those naturally associated with Gene 214 gene. Examples of workable combinations of cell lines and expression vectors are described in Sambrook *et al*, *Molecular Cloning. A Laboratory Manual*, 2nd Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) or  
20 Ausubel *et al*, *Current Protocols in Molecular Biology*, J. Wiley and Sons, NY (1992). Many useful vectors are known in the art and can be obtained from such vendors as Stratagene (*supra*), New England BioLabs, Beverly, MA, U.S.A, Promega Biotech, and other biotechnology product suppliers. Promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used in prokaryotic  
25 hosts. Useful yeast promoters include promoter regions for metallothionein, 3-phosphoglycerate kinase or other glycolytic enzymes such as enolase or glyceraldehyde-3-phosphate dehydrogenase, enzymes responsible for maltose and galactose utilization, and others. Vectors and promoters suitable for use in yeast expression are further described in EP 73,675A. Appropriate non-native mammalian promoters might include  
30 the early and late promoters from SV40 (Fiers *et al*, *Nature*, 273:113 (1978)) or promoters derived from murine Moloney leukemia virus, mouse tumor virus, avian

5 sarcoma viruses, adenovirus II, bovine papilloma virus or polyoma. In addition, the construct may be joined to an amplifiable gene (*e.g.*, DHFR) so that multiple copies of the gene may be made. For appropriate enhancer and other expression control sequences, see also *Enhancers and Eukaryotic Gene Expression*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1983). While such expression vectors may replicate autonomously,  
10 they may also replicate by being inserted into the genome of the host cell, by methods well known in the art.

Expression and cloning vectors will likely contain a selectable marker, a gene encoding a protein necessary for survival or growth of a host cell transformed with the vector. The presence of this gene ensures growth of only those host cells which express  
15 the inserts. Typical selection genes encode proteins that a) confer resistance to antibiotics or other toxic substances, *e.g.* ampicillin, neomycin, methotrexate, etc.; b) complement auxotrophic deficiencies, or c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for Bacilli. The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts  
20 are well known in the art.

The vectors containing the nucleic acids of interest can be transcribed *in vitro*, and the resulting RNA introduced into the host cell by well-known methods, *e.g.*, by injection (see, Kubo *et al*, *FEBS Letts.* 241:119 (1988)), or the vectors can be introduced directly into host cells by methods well known in the art, which vary depending on the type of  
25 cellular host, including electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent, such as a retroviral genome); and other methods. See generally, Sambrook *et al.*, 1989 and Ausubel *et al.*, 1992. The introduction of the nucleic acids into the host cell by  
30 any method known in the art, including those described above, will be referred to herein

5 as "transformation." The cells into which have been introduced nucleic acids described above are meant to also include the progeny of such cells.

Large quantities of the nucleic acids and proteins of the present invention may be prepared by expressing the Gene 214 nucleic acids or portions thereof in vectors or other expression vehicles in compatible prokaryotic or eukaryotic host cells. The most  
10 commonly used prokaryotic hosts are strains of *Escherichia coli*, although other prokaryotes, such as *Bacillus subtilis* or *Pseudomonas* may also be used.

Mammalian or other eukaryotic host cells, such as those of yeast, filamentous fungi, plant, insect, or amphibian or avian species, may also be useful for production of the proteins of the present invention. Propagation of mammalian cells in culture is per se  
15 well known. See, Jakoby and Pastan (eds.), *Cell Culture. Methods in Enzymology*, volume 58, Academic Press, Inc., Harcourt Brace Jovanovich, NY, (1979)). Examples of commonly used mammalian host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cells, and WI38, BHK, and COS cell lines, although it will be appreciated by the skilled practitioner that other cell lines may be appropriate, e.g., to provide higher  
20 expression desirable glycosylation patterns, or other features.

Clones are selected by using markers depending on the mode of the vector construction. The marker may be on the same or a different DNA molecule, preferably the same DNA molecule. In prokaryotic hosts, the transformant may be selected, e.g., by resistance to ampicillin, tetracycline or other antibiotics. Production of a particular  
25 product based on temperature sensitivity may also serve as an appropriate marker.

Prokaryotic or eukaryotic cells transformed with the nucleic acids of the present invention will be useful not only for the production of the nucleic acids and proteins of the present invention, but also, for example, in studying the characteristics of Gene 214 proteins.

30 Antisense nucleic acid sequences are useful in preventing or diminishing the expression of Gene 214 gene, as will be appreciated by one skilled in the art. For

5 example, nucleic acid vectors containing all or a fragment Gene 214 gene,  
complementary sequences of the former, or other sequences from the 12q23-qter region  
may be placed under the control of a promoter in an antisense orientation and introduced  
into a cell. Such fragments can be 16 or more nucleotides in length. Expression of such  
an antisense construct within a cell will interfere with Gene 214 transcription and/or  
10 translation and/or replication.

The probes and primers based on the Gene 214 gene sequences disclosed herein  
are used to identify homologous Gene 214 gene sequences and proteins in other species.  
These Gene 214 gene sequences and proteins are used in the diagnostic/prognostic,  
therapeutic and drug screening methods described herein for the species from which they  
15 have been isolated.

### III. PROTEIN EXPRESSION AND PURIFICATION

Expression and purification of the Gene 214 protein of the invention can be  
performed essentially as outlined below. To facilitate the cloning, expression and  
20 purification of membrane and secreted protein from the 12q23-qter, a gene expression  
system, such as the pET System (Novagen), for cloning and expression of recombinant  
proteins in *E. coli* is selected. Also, a DNA sequence encoding a peptide tag, the His-  
Tap, is fused to the 3' end of DNA sequences of interest to facilitate purification of the  
recombinant protein products. The 3' end is selected for fusion to avoid alteration of any  
25 5' terminal signal sequence.

Nucleic acids chosen, for example, from the nucleic acids set forth SEQ ID NO:2,  
SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO: 8, and SEQ ID NO:10, or SEQ ID NO:1 for  
cloning the genes are prepared by polymerase chain reaction (PCR). Synthetic  
oligonucleotide primers specific for the 5' and 3' ends of the nucleotide sequences are  
30 designed and purchased from Life Technologies (Gaithersburg, MD). All forward  
primers (specific for the 5' end of the sequence) are designed to include an *NcoI* cloning

5 site at the 5' terminus. These primers are designed to permit initiation of protein translation at the methionine residue encoded within the *NcoI* site followed by a valine residue and the protein encoded by the DNA sequence. All reverse primers (specific for the 3' end of the sequence) include an *EcoRI* site at the 5' terminus to permit cloning of the sequence into the reading frame of the pET-28b. The pET-28b vector provides a  
10 sequence encoding an additional 20 carboxyl-terminal amino acids including six histidine residues (at the C-terminus), which comprise the histidine affinity tag.

DNA prepared from the 12q23-qter region is used as the source of template DNA for PCR amplification (Ausubel *et al*, *Current Protocols in Molecular Biology*, John Wilty & Sons (1994)). To amplify a DNA sequence containing the nucleotide sequence,  
15 c DNA (50 ng) is introduced into a reaction vial containing 2 mM MgCl<sub>2</sub>, 1 micromolar synthetic oligonucleotide primers (forward and reverse primers) complementary to and flanking a defined 12q23-qter region, 0.2 mM of each of deoxynucleotide triphosphate, dATP, dGTP, dCTP, dTTP and 2.5 units of heat stable DNA polymerase (Amplitaq, Roche Molecular Systems, Inc., Branchburg, NJ) in a final volume of 100 microliters.

20 Upon completion of thermal cycling reactions, each sample of amplified DNA is purified using the Qiaquick Spin PCR purification kit (Qiagen, Gaithersburg, MD). All amplified DNA samples are subjected to digestion with the restriction endonucleases, *e.g.*, *NcoI* and *EcoRI* (New England BioLabs, Beverly, MA, U.S.A.) (Ausubel *et al*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)). DNA  
25 samples are then subjected to electrophoresis on 1.0% NuSeive (FMC BioProducts, Rockland, ME) agarose gels. DNA is visualized by exposure to ethidium bromide and long wave UV irradiation. DNA contained in slices isolated from the agarose gel are purified using the Bio 101 GeneClean Kit protocol (Bio 101, Vista, CA).

The pET-28b vector is prepared for cloning by digestion with restriction  
30 endonucleases, *e.g.*, *NcoI* and *EcoRI* (New England BioLabs, Beverly, MA) (Ausubel *et al*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)). The pET-

5 28a vector, which encodes the histidine affinity tag that can be fused to the 5' end of an inserted gene, is prepared by digestion with appropriate restriction endonucleases.

Following digestion, DNA inserts are cloned (Ausubel *et al*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)) into the previously digested pET-28b expression vector. Products of the ligation reaction are then used to transform the  
10 BL21 strain of *E. coli* (Ausubel *et al*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)) as described below.

Competent bacteria, *E. coli* strain BL21 or *E. coli* strain BL21 (DE3), are transformed with recombinant pET expression plasmids carrying the cloned sequence according to standard methods (Ausubel *et al*, *Current Protocols in Molecular Biology*,  
15 John Wiley & Sons, Inc. (1994)). Briefly, 1 microliter of ligation reaction is mixed with 50 microliters of electrocompetent cells and subjected to a high voltage pulse, after which samples were incubated in 0.45 ml SOC medium (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 20 mM glucose) at 37°C with shaking for 1 hour. Samples are then spread on LB agar plates containing 25 µg/ml  
20 kanamycin sulfate for growth overnight. Transformed colonies of BL21 are then picked and analyzed to evaluate cloned inserts, as described below.

Individual BL21 clones transformed with recombinant pET-28b 12q23-qter region nucleotide sequences are analyzed by PCR amplification of the cloned inserts using the same forward and reverse primers specific for the 12q23-qter region sequences  
25 that are used in the original PCR amplification cloning reactions. Successful amplification verifies the integration of the sequence in the expression vector (Ausubel *et al*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)).

Individual clones of recombinant pET-28b vectors carrying properly cloned 12q23-qter region nucleotide sequences are picked and incubated in 5 ml of LB broth  
30 plus 25 µg/ml kanamycin sulfate overnight. The following day plasmid DNA is isolated

5 and purified using the Qiagen plasmid purification protocol (Qiagen Inc., Chatsworth, CA).

The pET vector can be propagated in any *E. coli* K-12 strain, *e.g.*, HMS174, HB101, JM109, DH5 and the like, for purposes of cloning or plasmid preparation. Hosts for expression include *E. coli* strains containing a chromosomal copy of the gene for T7  
10 RNA polymerase. These hosts are lysogens of bacteriophage DE3, a lambda derivative that carries the *lacI* gene, the *lacUV5* promoter and the gene for T7 RNA polymerase. T7 RNA polymerase is induced by addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG), and the T7 RNA polymerase transcribes any target plasmid containing a functional T7 promoter, such as pET-28b, carrying its gene of interest. Strains include, for example, BL21(DE3)  
15 (Studier *et al*, *Meth. Enzymol.*, 185:60-89 (1990)).

To express the recombinant sequence, 50 ng of plasmid DNA are isolated as described above to transform competent BL21(DE3) bacteria as described above (provided by Novagen as part of the pET expression kit). The *lacZ* gene ( $\beta$ -galactosidase) is expressed in the pET-System as described for the 12q23-qter region  
20 recombinant constructions. Transformed cells were cultured in SOC medium for 1 hour, and the culture is then plated on LB plates containing 25  $\mu$ g/ml kanamycin sulfate. The following day, the bacterial colonies are pooled and grown in LB medium containing kanamycin sulfate (25  $\mu$ g/ml) to an optical density at 600 nm of 0.5 to 1.0 O.D. units, at which point 1 mM IPTG was added to the culture for 3 hours to induce gene expression  
25 of the 12q23-qter region recombinant DNA constructions.

After induction of gene expression with IPTG, bacteria are collected by centrifugation in a Sorvall RC-3B centrifuge at 3500 x g for 15 minutes at 4°C. Pellets are resuspended in 50 ml of cold mM Tris-HCl, pH 8.0, 0.1 M NaCl and 0.1 mM EDTA (STE buffer). Cells are then centrifuged at 2000 x g for 20 minutes at 4°C. Wet pellets  
30 are weighed and frozen at -80°C until ready for protein purification.

5 A variety of methodologies known in the art can be used to purify the isolated proteins (Coligan *et al*, *Current Protocols in Protein Science*, John Wiley & Sons (1995)). For example, the frozen cells can be thawed, resuspended in buffer and ruptured by several passages through a small volume microfluidizer (Model M-110S, Microfluidics International Corp., Newton, MA). The resultant homogenate is  
10 centrifuged to yield a clear supernatant (crude extract) and, following filtration, the crude extract is fractioned over columns. Fractions are monitored by absorbance at OD<sub>280</sub> nm and peak fractions may be analyzed by SDS-PAGE.

The concentrations of purified protein preparations are quantified spectrophotometrically using absorbance coefficients calculated from amino acid content  
15 (Perkins, *Eur. J. Biochem.*, 157:169-180 (1986)). Protein concentrations are also measured by the method of Bradford, *Anal. Biochem.*, 72:248-254 (1976) and Lowry *et al*, *J. Biol. Chem.*, 193:265-275 (1951) using bovine serum albumin as a standard.

SDS-polyacrylamide gels of various concentrations are purchased from BioRad (Hercules, CA), and stained with Coomassie blue. Molecular weight markers may  
20 include rabbit skeletal muscle myosin (200 kDa), *E. coli*  $\beta$ -galactosidase (116 kDa), rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), egg white lysozyme (14.4 kDa) and bovine aprotinin (6.5 kDa).

Proteins can also be isolated by other conventional means of protein biochemistry  
25 and purification to obtain a substantially pure product, *i.e.*, 80, 95, or 99% free of cell component contaminants, as described in Jacoby, *Methods in Enzymology*, Vol. 104, Academic Press, New York (1984); Scoopes, *Protein Purification, Principles and Practice*, 2<sup>nd</sup> Ed., Springer-Verlag, New York (1987); and Deutscher (ed.), *Guide to Protein Purification, Methods in Enzymology*, Vol. 182 (1990). If the protein is secreted,  
30 it can be isolated from the supernatant in which the host cell is grown; otherwise, it can be isolated from a lysate of the host cells.

5           Once a sufficient quantity of the desired protein has been obtained, it may be used  
for various purposes. One use of the protein or polypeptide is the production of  
antibodies specific for binding. These antibodies may be either polyclonal or  
monoclonal, and may be produced by *in vitro* or *in vivo* techniques well known in the art.  
Monoclonal antibodies to epitopes of any of the peptides identified and isolated as  
10 described can be prepared from murine hybridomas (Kohler, *Nature*, 256:495 (1975)). In  
summary, a mouse is inoculated with a few micrograms of protein over a period of two  
weeks. The mouse is then sacrificed. The cells that produce antibodies are then removed  
from the mouse's spleen. The spleen cells are then fused with polyethylene glycol with  
mouse myeloma cells. The successfully fused cells are diluted in a microtiter plate and  
15 growth of the culture is continued. The amount of antibody per well is measured by  
immunoassay methods such as ELISA (Engvall, *Meth. Enzymol.*, 70:419 (1980)). Clones  
producing antibody can be expanded and further propagated to produce protein  
antibodies. Other suitable techniques involve *in vitro* exposure of lymphocytes to the  
antigenic polypeptides, or alternatively, to selection of libraries of antibodies in phage or  
20 similar vectors. See Huse *et al*, *Science*, 246:1275-1281 (1989). For additional  
information on antibody production see Davis *et al*, *Basic Methods in Molecular Biology*,  
Elsevier, NY, Section 21-2 (1989). Such antibodies are particularly useful in diagnostic  
assays for detection of variant protein forms, or as an active ingredient in a  
pharmaceutical composition.

### 25           **III. TRANSFORMED HOSTS, DEVELOPMENT OF PHARMACEUTICALS AND RESEARCH TOOLS**

Cells and animals that carry Gene 214 can be used as model systems to study and  
test for substances that have potential as therapeutic agents.. The cells are typically  
30 cultured mesenchymal stem cells. These may be isolated from individuals with somatic  
or germline Gene 214. Alternatively, the cell line can be engineered to carry the Gene

214, as described above. After a test substance is applied to the cells, the transformed phenotype of the cell is determined. Any trait of transformed cells can be assessed, including respiratory diseases including asthma, atopy, and response to application of putative therapeutic agents.

#### IV. DIAGNOSTIC APPLICATIONS

As discussed herein, chromosomal region 12q23-qter has been genetically linked to a variety of diseases and disorders. This invention provides nucleic acids and SNPs which can be useful in diagnosing individuals with chromosomal abnormalities linked to these diseases.

*Antibody-based diagnostic methods:* The invention provides methods for detecting disease-associated antigenic components in a biological sample, which methods comprise the steps of: (i) contacting a sample suspected to contain a disease-associated antigenic component with an antibody specific for an disease-associated antigen, extracellular or intracellular, under conditions in which a stable antigen-antibody complex can form between the antibody and disease-associated antigenic components in the sample; and (ii) detecting any antigen-antibody complex formed in step (i) using any suitable means known in the art, wherein the detection of a complex indicates the presence of disease-associated antigenic components in the sample. It will be understood that assays that utilize antibodies directed against sequences previously unidentified, or previously unidentified as being disease-associated, which sequences are disclosed herein, are within the scope of the invention.

Many immunoassay formats are known in the art, and the particular format used is determined by the desired application. An immunoassay can use, for example, a monoclonal antibody directed against a single disease-associated epitope, a combination of monoclonal antibodies directed against different epitopes of a single disease - associated antigenic component, monoclonal antibodies directed towards epitopes of

5 different disease -associated antigens, polyclonal antibodies directed towards the same disease -associated antigen, or polyclonal antibodies directed towards different disease -associated antigens. Protocols can also, for example, use solid supports, or may involve immunoprecipitation.

Typically, immunoassays use either a labeled antibody or a labeled antigenic  
10 component (e.g., that competes with the antigen in the sample for binding to the antibody). Suitable labels include without limitation enzyme-based, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays that amplify the signals from the probe are also known, such as, for example, those that utilize biotin and avidin, and enzyme-labeled immunoassays, such as ELISA assays.

15 Kits suitable for antibody-based diagnostic applications typically include one or more of the following components:

(i) Antibodies: The antibodies can be pre-labeled; alternatively, the antibody may be unlabeled and the ingredients for labeling can be included in the kit in separate containers, or a secondary, labeled antibody is provided; and

20 (ii) Reaction components: The kit can also contain other suitably packaged reagents and materials needed for the particular immunoassay protocol, including solid-phase matrices, if applicable, and standards.

The kits referred to above can include instructions for conducting the test. Furthermore, in preferred embodiments, the diagnostic kits are adaptable to high-  
25 throughput and/or automated operation.

*Nucleic-acid-based diagnostic methods:* The invention provides methods for detecting disease -associated nucleic acids in a sample, such as in a biological sample, which methods comprise the steps of: (i) contacting a sample suspected to contain a disease -associated nucleic acid with one or more disease -associated nucleic acid probes  
30 under conditions in which hybrids can form between any of the probes and disease -associated nucleic acid in the sample; and (ii) detecting any hybrids formed in step (i)

5 using any suitable means known in the art, wherein the detection of hybrids indicates the presence of the disease -associated nucleic acid in the sample. To detect disease - associated nucleic acids present in low levels in biological samples, it may be necessary to amplify the disease -associated sequences or the hybridization signal as part of the diagnostic assay. Techniques for amplification are known to those of skill in the art.

10 Disease -associated nucleic acids useful as probes in diagnostic methods include oligonucleotides at least about 15 nucleotides in length, preferably at least about 20 nucleotides in length, and most preferably at least about 25-55 nucleotides in length, that hybridize specifically with one or more disease -associated nucleic acids.

15 A sample to be analyzed, such as, for example, a tissue sample, may be contacted directly with the nucleic acid probes. Alternatively, the sample may be treated to extract the nucleic acids contained therein. It will be understood that the particular method used to extract DNA will depend on the nature of the biological sample. The resulting nucleic acid from the sample may be subjected to gel electrophoresis or other size separation techniques, or, the nucleic acid sample may be immobilized on an appropriate solid  
20 matrix without size separation.

Kits suitable for nucleic acid-based diagnostic applications typically include the following components:

(i) *Probe DNA*: The probe DNA may be prelabeled; alternatively, the probe DNA may be unlabeled and the ingredients for labeling may be included in the kit  
25 in separate containers; and

(ii) *Hybridization reagents*: The kit may also contain other suitably packaged reagents and materials necessary or desirable for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards.

30 In cases where a disease condition is suspected to involve an alteration of the disease gene, specific oligonucleotides may be constructed and used to assess the level of disease mRNA in cells affected or other tissue affected by the disease.

5 For example, to test whether a person has a disease gene, polymerase chain  
reaction can be used. In order to identify an individual who possesses the disease gene or  
the wild type copy, two oligonucleotides are synthesized by standard methods or are  
obtained from a commercial supplier of custom-made oligonucleotides. The length and  
base composition are determined by standard criteria using the Oligo 4.0 primer Picking  
10 program (Wojchich Rychlik, 1992). One of the oligonucleotides is designed so that it  
will hybridize only to the disease gene DNA under the PCR conditions used. The other  
oligonucleotide is designed to hybridize to a segment of genomic DNA, wild type or non  
disease gene such that amplification of DNA using these oligonucleotide primers  
produces a conveniently identified DNA fragment. Tissue samples may be obtained from  
15 hair follicles, whole blood, or the buccal cavity. The DNA fragment generated by this  
procedure is sequenced by standard techniques.

Other amplification techniques besides PCR may be used as alternatives, such as  
ligation-mediated PCR or techniques involving Q-beta replicase (Cahill *et al*, *Clin.*  
*Chem.*, 37(9):1482-5 (1991)). Products of amplification can be detected by agarose gel  
20 electrophoresis, quantitative hybridization, or equivalent techniques for nucleic acid  
detection known to one skilled in the art of molecular biology (Sambrook *et al*,  
*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring,  
NY (1989)). Other alterations in the disease gene may be diagnosed by the same type of  
amplification-detection procedures, by using oligonucleotides designed to identify those  
25 alterations.

## V. GENOMIC SCREENING

The use of polymorphic genetic markers linked to the Gene 214 gene is very  
useful in predicting susceptibility to the diseases genetical linked to 12q23-qter.  
30 Similarly, as provided in Table 5 the identification of polymorphic genetic markers  
within the Gene 214 gene will allow the identification of specific allelic variants that are

5 in linkage disequilibrium with other genetic lesions that affect one of the disease states discussed herein including respiratory disorders and obesity. SSCP allows the identification of polymorphisms within the genomic and coding region of the disclosed gene. Table 3 provides primers which one skilled in the art could identify exons which contain SNP's. Table 4 provides primers to identify the sequence change. This  
10 information can assist one skilled in the art to identify additional SNP's for use in genomic screening.

This method has been used successfully by others skilled in the art (*e.g.*, Sheffield *et al*, *Genet.*, 4:1837-1844 (1995); LeBlanc-Straceski *et al*, *Genomics*, 19:341-9 (1994); Chen *et al*, *Genomics*, 25:1-8 (1995)). Use of these reagents with populations or  
15 individuals will predict their risk for diseases or disorders described herein, especially respiratory disorders and obesity.

## VI. TREATMENT OF DISORDERS

Thus, the present invention provides methods of screening for drugs comprising  
20 contacting such an agent with a novel protein of this invention or fragment thereof and assaying (i) for the presence of a complex between the agent and the protein or fragment, or (ii) for the presence of a complex between the protein or fragment and a ligand, by methods well known in the art. In such competitive binding assays the novel protein or fragment is typically labeled. Free protein or fragment is separated from that present in a  
25 protein:protein complex, and the amount of free (*i.e.*, uncomplexed) label is a measure of the binding of the agent being tested to the novel protein or its interference with protein ligand binding, respectively.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of specifically binding the Gene 214  
30 protein compete with a test compound for binding to the Gene 214 protein or fragments

5 thereof. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants of a Gene 214 protein.

The goal of rational drug design is to produce structural analogs of biologically active proteins of interest or of small molecules with which they interact (*e.g.*, agonists, antagonists, inhibitors) in order to fashion drugs which are, for example, more active or  
10 stable forms of the protein, or which, *e.g.*, enhance or interfere with the function of a protein *in vivo*. See, *e.g.*, Hodgson, *Bio/Technology*, 9:19-21 (1991). In one approach, one first determines the three-dimensional structure of a protein of interest or, for example, of the Gene 214 receptor or ligand complex, by x-ray crystallography, by computer modeling or most typically, by a combination of approaches. Less often, useful  
15 information regarding the structure of a protein may be gained by modeling based on the structure of homologous proteins. An example of rational drug design is the development of HIV protease inhibitors (Erickson *et al*, *Science*, 249:527-533 (1990)). In addition, peptides (*e.g.*, Gene 214 protein) are analyzed by an alanine scan (Wells, *Methods in Enzymol.*, 202:390-411 (1991)). In this technique, an amino acid residue is replaced by  
20 Ala, and its effect on the peptide's activity is determined. Each of the amino acid residues of the peptide is analyzed in this manner to determine the important regions of the peptide.

It is also possible to isolate a target-specific antibody, selected by a functional assay, and then to solve its crystal structure. In principle, this approach yields a  
25 pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analog of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically  
30 or biologically produced banks of peptides. Selected peptides would then act as the pharmacore.

5           Thus, one may design drugs which have, *e.g.*, improved Gene 214 proteinactivity or stability or which act as inhibitors, agonists, antagonists, etc. of Gene 214 proteinactivity. By virtue of the availability of cloned Gene 214 gene sequences, sufficient amounts of the Gene 214 protein may be made available to perform such analytical studies as x-ray crystallography. In addition, the knowledge of the Gene 214  
10 protein sequence will guide those employing computer modeling techniques in place of, or in addition to x-ray crystallography.

          Cells and animals that carry the Gene 214 gene or an analog thereof can be used as model systems to study and test for substances that have potential as therapeutic agents. After a test substance is applied to the cells, the transformed phenotype of the cell  
15 is determined.

          The therapeutic agents and compositions of the present invention are useful for preventing or treating respiratory disease. Pharmaceutical formulations suitable for therapy comprise the active agent in conjunction with one or more biologically acceptable carriers. Suitable biologically acceptable carriers include, but are not limited to,  
20 phosphate-buffered saline, saline, deionized water, or the like. Preferred biologically acceptable carriers are physiologically or pharmaceutically acceptable carriers.

          The compositions include an effective amount of active agent. Effective amounts are those quantities of the active agents of the present invention that afford prophyladic protection against a respiratory disease, or which result in amelioration or cure of an  
25 existing respiratory disease. Prophylactic methods incorporate a prophylactically effective amount of an active agent or composition. A prophylactically effective amount is an amount effective to prevent disease. Treatment methods incorporate a therapeutically effective amount of an active agent or composition. A therapeutically effective amount is an amount sufficient to ameliorate or eliminate the symptoms of  
30 disease. The effective amount will depend upon the agent, the severity of disease and the nature of the disease, and the particular host. The amount can be determined by

5 experimentation known in the art, such as by establishing a matrix of dosage amounts and frequencies of dosage administration and comparing a group of experimental units or subjects to each point in the matrix. The prophylactically and/or therapeutically effective amounts can be administered in one administration or over repeated administrations. Therapeutic administration can be followed by prophylactic administration, once initial  
10 clinical symptoms of disease have been resolved.

The agents and compositions can be administered topically or systemically. Systemic administration includes both oral and parental routes. Parental routes include, without limitation, subcutaneous, intramuscular, intraperitoneal, intravenous, transdermal, and intranasal administration.

## 15 VII. GENE THERAPY

In recent years, significant technological advances have been made in the area of gene therapy for both genetic and acquired diseases. (Kay et al, *Proc. Natl. Acad. Sci. USA*, 94:12744-12746 (1997)) Gene therapy can be defined as the deliberate transfer of  
20 DNA for therapeutic purposes. Improvement in gene transfer methods has allowed for development of gene therapy protocols for the treatment of diverse types of diseases. Gene therapy has also taken advantage of recent advances in the identification of new therapeutic genes, improvement in both viral and nonviral gene delivery systems, better understanding of gene regulation, and improvement in cell isolation and transplantation.  
25 Gene therapy would be carried out according to generally accepted methods as described by, for example, Friedman, *Therapy for Genetic Diseases*, Friedman, Ed., Oxford University Press, pages 105-121 (1991).

Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art, and any suitable vector may be  
30 used. Methods for introducing DNA into cells such as electroporation, calcium phosphate co-precipitation, and viral transduction are known in the art, and the choice of

5 method is within the competence of one skilled in the art (Robbins, Ed., *Gene Therapy Protocols*, Human Press, NJ (1997)). Cells transformed with a Gene 214 gene can be used as model systems to study chromosome 12 disorders and to identify drug treatments for the treatment of such disorders.

Gene transfer systems known in the art may be useful in the practice of the gene  
10 therapy methods of the present invention. These include viral and nonviral transfer methods. A number of viruses have been used as gene transfer vectors, including polyoma, *i.e.*, SV40 (Madzak *et al*, *J. Gen. Virol.*, 73:1533-1536 (1992)), adenovirus (Berkner, *Curr. Top. Microbiol. Immunol.*, 158:39-61 (1992); Berkner *et al*, *Bio Techniques*, 6:616-629 (1988); Gorziglia *et al*, *J. Virol.*, 66:4407-4412 (1992); Quantin *et al*, *Proc. Natl. Acad. Sci. USA*, 89:2581-2584 (1992); Rosenfeld *et al*, *Cell*, 68:143-155  
15 (1992); Wilkinson *et al*, *Nucl. Acids Res.*, 20:2233-2239 (1992); Stratford-Perricaudet *et al*, *Hum. Gene Ther.*, 1:241-256 (1990)), vaccinia virus (Mackett *et al*, *Biotechnology*, 24:495- 499 (1992)), adeno-associated virus (Muzyczka, *Curr. Top. Microbiol. Immunol.*, 158:91- 123 (1992); Ohi *et al*, *Gene*, 89:279-282 (1990)), herpes viruses including HSV and EBV (Margolskee, *Curr. Top. Microbiol. Immunol.*, 158:67-90 (1992); Johnson *et al*, *J. Virol.*, 66:2952-2965 (1992); Fink *et al*, *Hum. Gene Ther.*, 3:11-19 (1992); Breakfield *et al*, *Mol. Neurobiol.*, 1:337-371 (1987); Fresse *et al*, *Biochem. Pharmacol.*, 40:2189-2199 (1990)), and retroviruses of avian (Brandyopadhyay *et al*, *Mol. Cell Biol.*, 4:749-754 (1984); Petropoulos *et al*, *J. Virol.*, 66:3391-3397 (1992)), murine (Miller, *Curr.*  
20 *Top. Microbiol. Immunol.*, 158:1-24 (1992); Miller *et al*, *Mol. Cell Biol.*, 5:431- 437 (1985); Sorge *et al*, *Mol. Cell Biol.*, 4:1730-1737 (1984); Mann *et al*, *J. Virol.*, 54:401-407 (1985)), and human origin (Page *et al*, *J. Virol.*, 64:5370-5276 (1990); Buchsachaler *et al*, *J. Virol.*, 66:2731- 2739 (1992)). Most human gene therapy protocols have been based on disabled murine retroviruses.

30 Nonviral gene transfer methods known in the art include chemical techniques such as calcium phosphate coprecipitation (Graham *et al*, *Virology*, 52:456-467 (1973);

5 Pellicer *et al*, *Science*, 209:1414-1422 (1980)), mechanical techniques, for example microinjection (Anderson *et al*, *Proc. Natl. Acad. Sci. USA*, 77:5399-5403 (1980); Gordon *et al*, *Proc. Natl. Acad. Sci. USA*, 77:7380-7384 (1980); Brinster *et al*, *Cell*, 27:223-231 (1981); Constantini *et al*, *Nature*, 294:92-94 (1981)), membrane fusion-mediated transfer via liposomes (Felgner *et al*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7417  
10 (1987); Wang *et al*, *Biochemistry*, 28:9508-9514 (1989); Kaneda *et al*, *J. Biol. Chem.*, 264:12126-12129 (1989); Stewart *et al*, *Hum. Gene Ther.*, 3:267-275 (1992); Nabel *et al*, *Science*, 249:1285-1288 (1990); Lim *et al*, *Circulation*, 83:2007-2011 (1992)), and direct DNA uptake and receptor-mediated DNA transfer (Wolff *et al*, *Science*, 247:1465-1468 (1990); Wu *et al*, *BioTechniques*, 11:474-485 (1991); Zenke *et al*, *Proc. Natl. Acad. Sci. USA*, 87:3655-3659 (1990); Wu *et al*, *J. Biol. Chem.*, 264:16985-16987 (1989); Wolff *et al*, *BioTechniques*, 11:474-485 (1991); Wagner *et al*, 1990; Wagner *et al*, *Proc. Natl. Acad. Sci. USA*, 88:4255-4259 (1991); Cotten *et al*, *Proc. Natl. Acad. Sci. USA*, 87:4033-4037 (1990); Curiel *et al*, *Proc. Natl. Acad. Sci. USA*, 88:8850-8854 (1991); Curiel *et al*, *Hum. Gene Ther.*, 3:147-154 (1991)).

20 In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein, and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization, and degradation of the endosome before the coupled  
25 DNA is damaged.

Liposome/DNA complexes have been shown to be capable of mediating direct *in vivo* gene transfer. While in standard liposome preparations the gene transfer process is non-specific, localized *in vivo* uptake and expression have been reported in tumor deposits, for example, following direct *in situ* administration (Nabel, *Hum. Gene Ther.*,  
30 3:399-410 (1992)).

5 **VIII. TRANSGENIC ANIMALS**

This invention further relates to nonhuman transgenic animals capable of expressing an exogenous or non-naturally occurring variant Gene 214 gene. Such a transgenic animal can also have one or more endogenous genes inactivated or can, instead of expressing an exogenous variant gene, have one or more endogenous analogs  
10 inactivated. Any nonhuman animal can be used; however typical animals are rodents, such as mice, rats, or guinea pigs.

Animals for testing therapeutic agents can be selected after treatment of germline cells or zygotes. Thus, expression of an exogenous Gene 214 gene or a variant can be achieved by operably linking the gene to a promoter and optionally an enhancer, and then  
15 microinjecting the construct into a zygote. See, e.g., Hogan, *et al.*, *Manipulating the Mouse Embryo, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Such treatments include insertion of the exogenous gene and disrupted homologous genes. Alternatively, the gene(s) of the animals may be disrupted by insertion or deletion mutation of other genetic alterations using conventional techniques,  
20 such as those described by, for example, Capecchi, *Science*, 244:1288 (1989); Valancuis et al, *Mol. Cell Biol.*, 11:1402 (1991); Hasty et al, *Nature*, 350:243 (1991); Shinkai et al, *Cell*, 68:855 (1992); Mombaerts et al, *Cell*, 68:869 (1992); Philpott et al, *Science*, 256:1448 (1992); Snouwaert et al, *Science*, 257:1083 (1992); Donehower et al, *Nature*, 356:215 (1992). After test substances have been administered to the animals, modulation  
25 of the disorder must be assessed. If the test substance reduces the incidence of the disorder, then the test substance is a candidate therapeutic agent. These animal models provide an extremely important vehicle for potential therapeutic products.

The disclosure of each of the patents, patent applications and publications cited in the specification is hereby incorporated by reference herein in its entirety.

30 Although the invention has been set forth in detail, one skilled in the art will recognize that numerous changes and modifications can be made, and that such changes

- 5 and modifications may be made without departing from the spirit and scope of the invention.